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THE BASIC SCIENCE RESEARCH LABORATORY
A DIVISION OF
THE INSTITUTE OF SCIENTIFIC RESEARCH
UNIVERSITY OF CINCINNATI

UNIVERSITY OF CINCINNATI

Institute of Scientific Research

CORRELATED INVESTIGATIONS
—IN THE—
BASIC SCIENCES

A Series of Ten Reports from the
Basic Science Research Laboratory

Series IV, Number 2

March, 1928

STAFF OF
INSTITUTE OF SCIENTIFIC RESEARCH

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FOREWORD

To the best of our knowledge, there are ninety-two chemical elements. All matter everywhere, whether protoplasm or comet, is made up of some of these elements, which in turn are combinations of protons and electrons. More basically, then, all matter is composed of protons and electrons.

Our universe is a universe of law. An atom of hydrogen obeys the laws governing an atom of hydrogen whether it is in a starfish or in a star. Its location never dislocates the law. The laws governing the actions of protons and electrons are absolute through all space. They never change, never vacillate, never vary. They act with one hundred per cent perfection one hundred per cent of the time. They were in the beginning, are now, and ever shall be.

Fundamentally, therefore, all actions of matter and between matter must conform with the laws which control the actions of protons and electrons. Stated another way, all actions of matter are the results of the operation of a few laws (or perhaps, and more probably, one law!)

There is talk of a "vital" or "psychic" element. If there is suc an element, it too must obey law, and its law or laws could not l disharmonious with the laws of the protons and electrons. In modern science, conflicts in basic laws are inconceivable.

The physicist's conceptions of matter now begin with positive and negative electrical charges,—two intangible, non-solid bits of "energy," as intangible as any conception of a vital force. The interactions of protons and electrons create all matter and all of its manifestations. So the conception of a "vital" or "psychic" force, which must be an "energy" concept, could easily be considered as another form of the fundamental energy concept of physics.

At any rate, "vital" force as a separate factor in the control of the actions of the ninety-two elements, is in a nebulous and speculative state, whereas the basic laws governing matter are partly known. And certainly it is safe to assume that atoms and molecules obey the laws governing them, regardless of their location.

Logically then, the laws of the atoms and molecules must hold for atoms and molecules in biological processes.* This means that all science gets back to the same set of basic concepts and basic laws;

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*My own conception is that "life" is a particular state of matter in accordance with these laws.

that there are not many sciences (physics, chemistry, bio-chemistry, biology, astronomy, geology) but only one science, that the fundamental point of attack to obtain truth, is at the source of all actions; that a basic science laboratory would necessarily include in its personnel, workers from all the fields of science about which we have stupidly built fences, that the work of the personnel would be coordinated (the fences knocked down); and that the guiding motive should be to learn the truth, state it in its simplest expression, and indicate its applications.

It was upon such ideas as these that The Basic Science Research Laboratory of the University of Cincinnati was established in September, 1924. Obviously a point of beginning was to show by laboratory experiment that the laws and theories pertaining to atoms and molecules do hold in certain biological processes. One of these theories is the Quantum Theory. If the Quantum Theory holds in biology, then, it was argued, the killing of bacteria, the dissociation of enzymes, and similar actions by electromagnetic waves must take place *at critical points.* This was found to be so and the results were published.[1]

The papers herein presented embody some of the results of the work of the academic years 1926-1927. In each case the paper is written by the staff member who did most or all of the work on the particular subject. It should be recalled, however, that all of this work is coordinated. Individual credit is incidental; personal vanity has rarely promoted real research,—the real scientist is usually appalled at his little knowledge rather than exalted over it.

The personnel of the laboratory (June, 1927) is as follows:

George Sperti, *Director*

George N. Burger
John C. Fardon
John R. Loofbourow
Harry L. Miller
Curtis P. Mitchell
Leonora Neuffer
Robert J. Norris
Elizabeth Shelow
Harold Thomas
Dare A. Wells
Mary Alice Wells
Robert Withrow

It is hoped that a plant physiologist can be added to the staff during the year 1927-1928, to begin studies in botany analogous to those already under way in zoology.

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[1] Schneider and Sperti, "The Quantum Theory in Biology," Series IV, Paper 1; Institute of Scientific Research, University of Cincinnati, (April, 1926).

The addition of a philosopher is also contemplated in the near future for the various teleological philosophies, which have all been built up patiently and successively, have been knocked down by scientific discoveries. It would seem wiser to build from the scientifically known and to test by scientific methods.

In due time also, the other sciences,—geology, psychology, astronomy— will have at least liason researchers working part time in the Basic Science Research Laboratory and in their own departments.

HERMAN SCHNEIDER,
Director Institute of Scientific Research.

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INTRODUCTION

The major problem of the Basic Science Research Laboratory, at the present time, is to establish connections between the fundamental laws of the sciences, and in particular the application of the laws of physics to biology. The method includes theoretical as well as experimental investigations.

Obviously such procedure involves the solution of a number of seemingly isolated problems. For example, the lack of adequate methods for the chemical analyses of proteins necessitated spectroscopic investigation into the structure of molecules. Need of a galvanometer of proper sensitivity combined with the desired stability for some of the spectroscopic work led to the development of the instrument described in this pamphlet.

In order to arrive at absorption more fundamentally, theoretical investigations as to its nature have been carried on. Some of these are presented herein.

An attempt to study from the physicist's as well as the bio-chemist's viewpoint the action of X-rays and β-particles on tumors led to an investigation of the relative toxicities of various groupings of atoms and a study of their X-ray absorption.

In a similar manner many other problems which, at first sight, appeared to bear no relation to one another have been attempted. Further, as research progressed, possibilities of applications to problems other than those originally begun, presented themselves; for example, problems such as the preparation of vaccines through the aid of filtered ultra-violet radiations and the sterilization of enzymes by similar methods came to our attention.

Many problems such as these are under way in the laboratory. A few of those which have advanced sufficiently for publication of results are presented in this pamphlet.

Both theoretical considerations and laboratory indications have marked out definite lines of procedure in addition to those shown in the first paper from this laboratory* together with those herein published. All of these follow the general thesis of the laboratory or are fundamental thereto.

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*Schneider and Sperti, "The Quantum Theory in Biology," University of Cincinnati, Institute of Scientific Research, Series 4, Paper 1, April 2, 1926.

Some of the suggested lines are as follows:

1. Study of the structure of complex organic compounds through the aid of ultra-violet and infra-red spectroscopy.

2. Study of the response of nerves to electrical impulses.

3. Study of the mode of transmission of nerve impulses.

4. Quantitative as well as qualitative biological work in the long X-ray region—order of one Angstrom unit.

5. Sterilization of food products by means of filtered ultra-violet without subsequent destruction of any of the desired components.

6. Absorption of mechanical vibrations by chemical compounds as a function of the structure of the compound.

7. Use of high atomic weight elements in conjunction with X-rays in the treatment of tuberculosis and other diseases.

8. Focusing of X-rays for use in deep therapy.

9. Relations between charge, valence, ionic potential, and toxicity of the metals, etc.

10. Study of the action of ultra-violet on various portions of individual cells.

George Sperti,
Director Basic Science Research Laboratory.

THEORETICAL AND EXPERIMENTAL INVESTIGATION OF THE EXCITATION OF CORPUSCULAR, ATOMIC, AND MOLECULAR RADIATIONS IN TUMOR TISSUE

By George Sperti and Robert J. Norris

The agent which, in all radiations, produces biological effects, as in the case of cancer treatment, has never been definitely determined, notwithstanding the considerable work done on the subject.

Many theories have been advanced to explain the effects of radiations on cells, but in no case are sufficient data available to establish the truth of these theories. Although many experiments indicate that the ionizing power of a radiation is a measure of its biological effectiveness, this still remains to be proved. The only authentic information we have on the subject is that the energy absorbed is to some extent a measure of the effectiveness of a radiation.

Two methods may be employed for increasing the physiological effect of radiations:

1. Sensitization.
2. Increased absorption.

In the case of sensitization, the action may be attributed either to an increased or decreased rate of metabolism of the tissue.

E. G. Mayer[1], for instance, has indicated that an administration of an hypertonic solution of dextrose increases the sensitivity of tumor tissue to radiations.

Inasmuch as the energy absorbed is a measure of the biological effectiveness of radiations, it seems that methods for increasing the absorption may prove beneficial.

It is this phase of the problem in which we are primarily interested.

I

STATEMENT OF PROBLEM

In the X-ray treatment of cancer, too much consideration has been given to the energy *incident at* the surface of the cancer and not sufficient emphasis is placed on the amount of energy *absorbed by* the cancer. The problem of curing deep-seated growths by the use of

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[1] Mayer, E. G., Strahlentherapie, XXIII, 604-630 (1926) also translated, Radiology, St. Paul, 14-16, VII (1926).

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[1] Mayer, E. G., Strahlentherapie, XXIII, 604-630 (1926) also translated, Radiology, St. Paul, 14-16, VII (1926).

It is well known that if radiations of comparatively long wave-length (soft X-rays) are used in treating deep-seated cancers, most of the energy will be absorbed before it reaches the desired spot; consequently very little will remain to be absorbed by the cancer. On the other hand, if the radiations are of such a nature that they will not be absorbed before reaching the tumor (hard X-rays, *i. e.* short radiations), it is likewise true that they will not be absorbed by the tumor. It seems, therefore, that for each case treated, there may be some critical wave-length which will produce best results. In other words, by using the proper wave-length, a greater percentage of the energy incident at the surface of the patient will be absorbed by the tumor than if any other wave-length were used.

This problem may be solved mathematically. It is one of determining the proper wave-length of X-rays to be used so that the percentage of the total energy incident at the surface of the patient, absorbed by the tumor, will have its maximum value.

Since there are two types of X-ray absorption (fluorescent and scattering) it is necessary to determine the relative values of each.

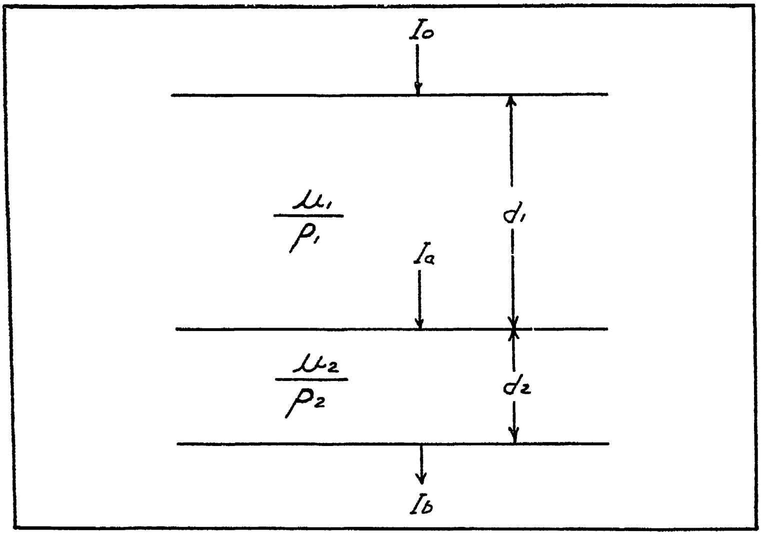

DIAGRAM A

Referring to the well-known law of absorption—

(1) $$I_d = I_o e^{-(\tau + \sigma) d} \text{ (see diagram above)}$$

in which I_o is the energy incident at the surface of the patient, I_d the energy at any depth d, τ the fluorescent absorption coefficient, and σ the scattering coefficient, we are able to determine how each type varies with λ and N, for—

$$(2) \qquad \tau = \frac{C\,\lambda^3\,N^4}{A}\,\rho \text{ and } \sigma = 0.402\,\frac{N}{A}\,\rho$$

where C is a constant, λ the wave-length of the incident radiation, N the atomic number of the absorber, A its atomic weight and ρ the density Since the amount of energy fluorescently absorbed and the amount of energy scattered may be determined independently as well as in combination, it is possible to determine which of these absorptions is responsible for physiological effects.

II

EFFECTIVE PART OF ENERGY

1. If the *total energy absorbed*[*] is effective in producing biological effects, the destruction of the cancer by radiations of different wave-lengths should follow the curve of total energy absorbed, plotted against wave-length. The equation for this curve may be derived as shown in Part I of the appended calculations (page 29). If, for a given depth and thickness of tumor, we plot α_2, *i. e.* $\frac{I_a - I_b}{I_o}$, against the wave-length of the incident radiation, the resulting curve will give the percentage of the total incident energy absorbed by the cancer for any wave-length. The point of the curve at which maximum absorption of energy occurs is given by equation (4) (page 30). Such a curve (μ) is seen in Figure I (page 14), the values used being given on the graph.

2. If *that part of the absorbed energy which is scattered*[**] is the only energy effective biologically, the destruction of the tumors by radiations of different wave-lengths should follow the curve of the scattered energy plotted against wave-length. The equation for the energy scattered in the tumor may be derived as shown in Part 2 (page 30) of the calculations.

If this equation is plotted using $d_1 = 4$ cm. and $d_2 = 2$ cm., the curve will be as represented in Figure I, marked σ.

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[*] All results are for monochromatic radiations.

[**] Scattering has been considered constant for all wave-lengths.

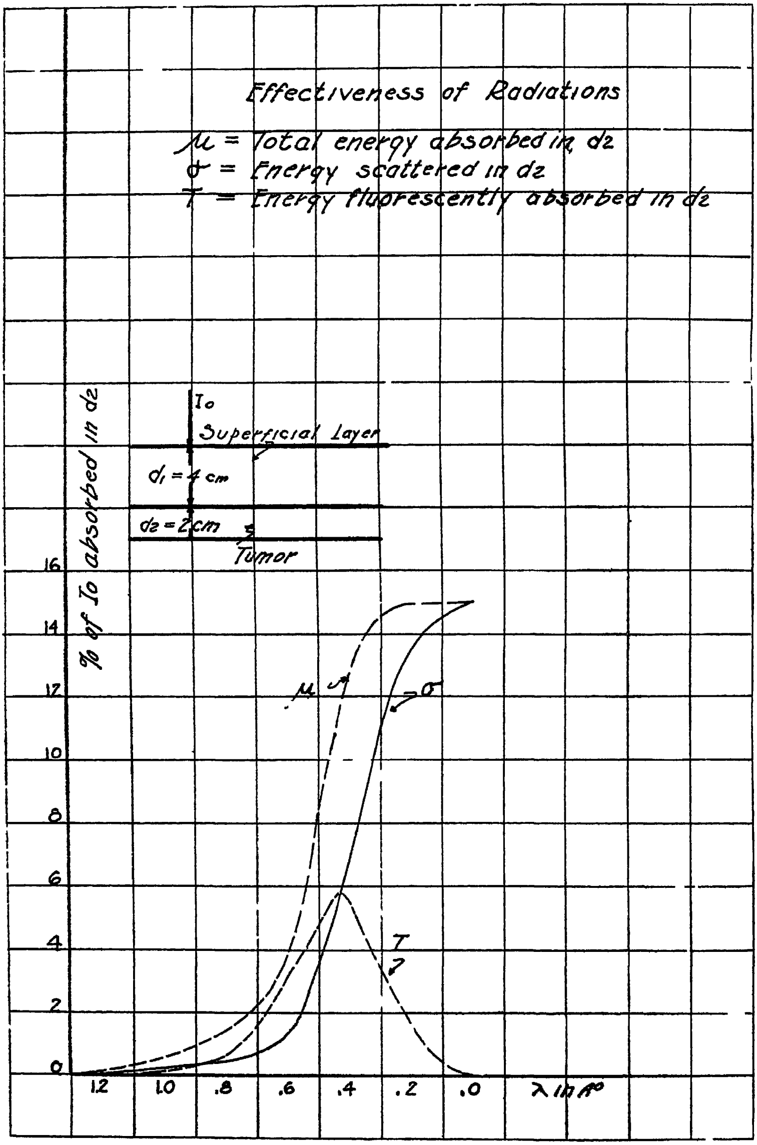

FIGURE I

3. If the *fluorescently absorbed energy* is effective in producing biological changes, the destruction of the tumor by radiations of different wave-lengths should follow the curve of the fluorescently

absorbed energy plotted against wave-length. The calculations for the energy fluorescently absorbed by the tumor are given in Part 3 (page 31).

If we plot the equation as in the previous cases, using the same values for d_1 and d_2, the curve takes on the form shown in Figure I, (τ). It has a surprisingly sharp maximum at 0.45 A° which gives an absorption of 5.9% of the total primary beam. At 0.6 A°, we have an absorption of but 3.1%, while at 0.1 A° we have an absorption of only 0.3%. Thus, in the case in which the patient is treated with a radiation of 0.6 A°. we have only 53% of the total possible absorption; and at 0.1 A° only 5% of the possible absorption. The maximum is given by equation (8) (page 31).

The three curves explained above and shown in Figure I are plotted to the same scale. A study of the curves in connection with the known biological reactions gives some clue as to which one of the three most nearly corresponds to the biological reactions.

Curves μ and σ, representing respectively the total energy absorbed and the energy scattered in the tumor, reach a maximum after which they remain practically constant for decreasing wave-lengths. If biological effects are due to these energies, they must follow the same curve, *i. e.*, with a constant energy input they must increase as the wave-length decreases to a certain value, after which they will remain practically constant.

It is well-known, however, that after certain wave-lengths are reached (the energy input remaining constant) further decreases in wave-length are accompanied by decreases in biological effect. Curve τ, representing fluorescent absorption, is the only one which shows this tendency.

From the foregoing discussion, it may be concluded that the part of the energy fluorescently absorbed probably represents the most effective portion of the absorbed energy. If such be the case, there is a limited band of wave-lengths within which radio-therapy treatments should be given.

III

MOST DESIRABLE WAVE-LENGTHS

Wave-lengths, which are shorter than the one giving maximum absorption in the cancer, will, with increased energy input, give equal fluorescent absorptions in the cancer, d_2 (diagram, page 12) with less fluorescent absorption in the superficial layer, d_1.

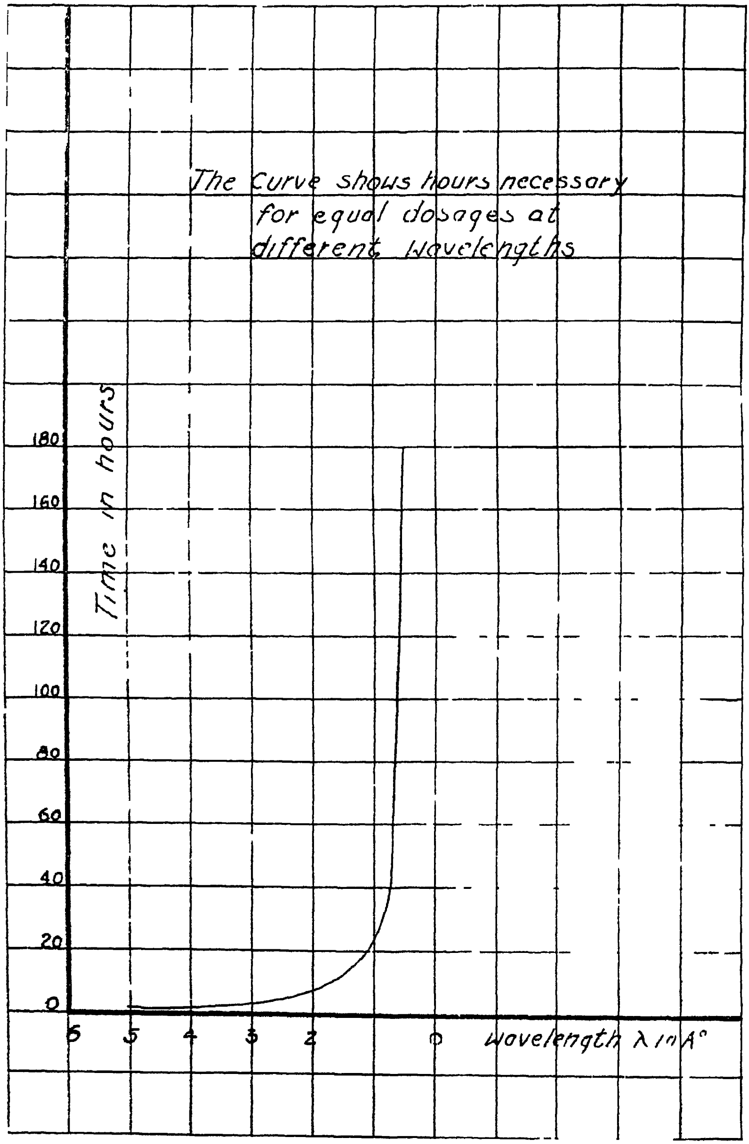

FIGURE II

In Figure II (above) are plotted the hours necessary to give fluorescent absorptions of equal amounts of energy in d_2 at various wave-lengths in comparison with that delivered by one hour exposure at 0.45 A°, the optimum wave-length. From the curve, it is seen that if, instead of treating the cancer with a radiation of 0.45 A°, we treat it with a radiation of 0.06 A°, it becomes necessary to give about one hundred times the dose to the patient at the port of entry in order to have the fluorescent absorptions in the cancer equal in both cases.

It must therefore be concluded that the most effective wave-length lies within a definite band of frequencies. The exact wave-length to be used will be determined by the biological reactions of the radiations, and the depth and thickness of the cancer. For example, in the case cited at the beginning of the paper, we have a choice between a treatment, say for one hour with a radiation of 0.45 A° or 3.3 hours with 0.3 A°. The energy fluorescently absorbed, however, in the superficial layer in the former will be 42% of the incident energy while in the latter only 16%.

The data used in determining the absorption coefficients are not sufficiently reliable to permit the use of the curves for quantitative work.

Equal absorptions of X-rays of different wave-lengths are often said not to produce the same biological effects. Some light may be thrown on this subject by considering the distribution of energy throughout a layer of homogeneous material.

Variation with Depth.

Figure III (page 18) shows the variation, with depth, of the amount of energy fluorescently absorbed in flesh for 1 A° and for 0.1 A°. Although at a certain depth, d, the total fluorescent absorption in the two cases may be equal, the energy absorbed in a thickness, dx, is not only different at various depths, but is also different for various wave-lengths at the same depth. In other words, at different wave-lengths, by properly proportioning the energy output of a tube, we may in equal periods of time produce the same fluorescent absorption in a given thickness of material, d, and yet have the absorption per thickness, dx, entirely different.

Since it is the energy absorbed *per cell* in which we are interested, the absorption per depth, dx, is of prime importance. For comparative data then it is quite essential that the absorption per cell, or layer of cells, be considered rather than the mass absorption (energy absorbed in the total depth, d). On this basis it seems quite possible that equal absorptions of energy of different wave-lengths will give identical results. (The X-ray region only is considered.)

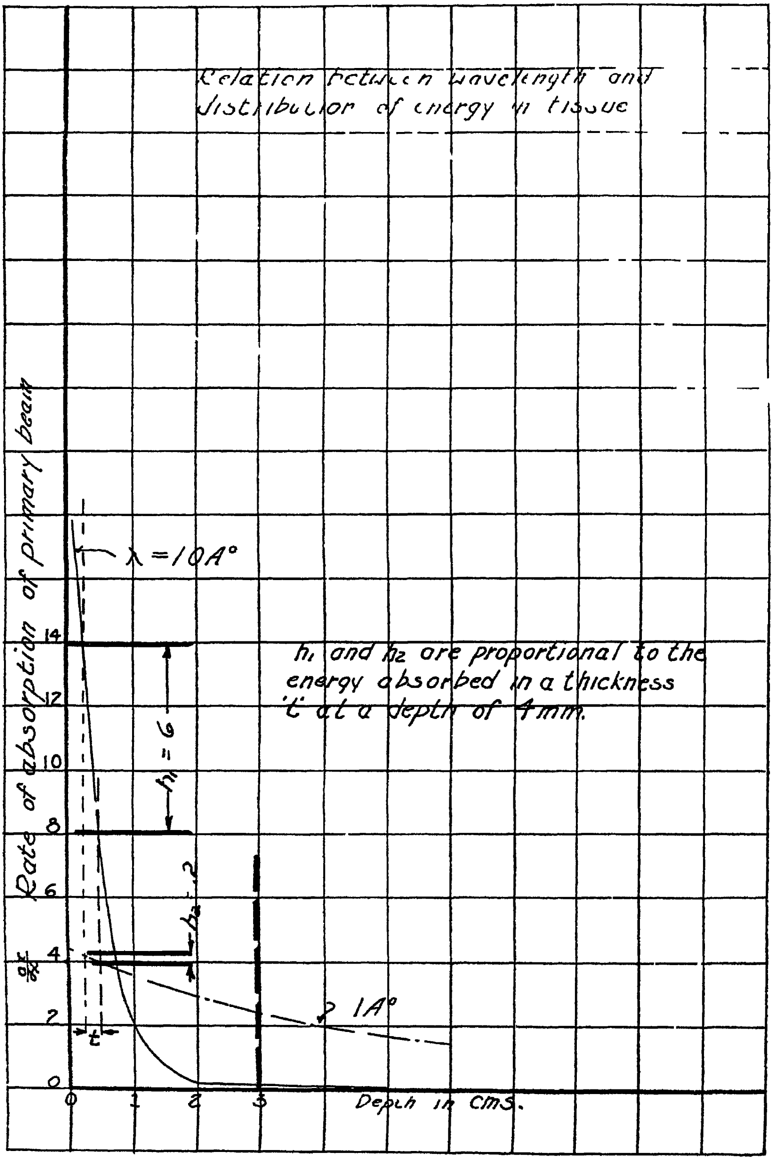

FIGURE III

Control of Absorption

One of the well-known difficulties in the treatment of cancer by means of X-rays is the prevention of lethal doses to normal cells and, at the same time, the destruction of malignant cells.

The difficulty of greater absorption of energy at the surface of the body than at the cancer (see Equation 1. page 12) could be met either by decreasing the absorption of energy by the superficial layer of tissue or by increasing the absorption by the tumor in order to have conditions more ideal. The latter method seems the more simple.

The method consists of converting, at the cancer, the energy of hard radiations, which are necessary for the penetration of the superficial layer, into energy of soft X-rays and β-particles, both of which are easily absorbed by the cancer.

As stated in one of the foregoing paragraphs, when X-rays pass through a medium, part of their energy is absorbed and changed to characteristic radiations, *i. e.*, characteristic of the absorbing medium. The wave-lengths of these radiations, for any given absorption band, are a function only of the atomic numbers of the elements of the medium.

In Equation 2 (page 13), the constant, C, depends upon the absorption band in which the incident radiation lies. It is seen from this equation that the amount of energy absorbed varies as the fourth power of the atomic number and the cube of the wave-length. This suggests a means for increasing the effectiveness of short radiations in radio-therapy. This method consists of the introduction of chemical elements into the cancer in order to increase indirectly the energy absorbed by the cancer (This method is discussed in full in the article, "A Comparative Study of Toxicities of Certain Groups of Chemical Substances Used in Cancer Treatment," page 32) It must be understood that the element used must be selected with due regard to the wave-length to be employed.

According to Stokes' Law, unless the exciting radiation is of a shorter wave-length than that characteristic of the absorbing medium, the characteristic radiation will not be emitted. In order to be useful, the characteristic radiations excited must be sufficiently long to be absorbed easily by the cancer; if not, they will be of no value in effecting a cure.

In addition to the characteristic radiations given off by an element, photo-electrons or β-particles are usually emitted in abundance.

Colwell and Russ[1] have shown that β-particles are among the most effective agents in radium for the treatment of cancer. Hence, the injection of elements of the proper atomic number into a tumor in connection with irradiation by X-rays of the proper wave-length should give results very similar to those which could be expected from finely divided radium scattered throughout a cancer.

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[1] Colwell and Russ, X-rays, Radium and The Living Cell, G. Bell & Sons, Ltd., London.

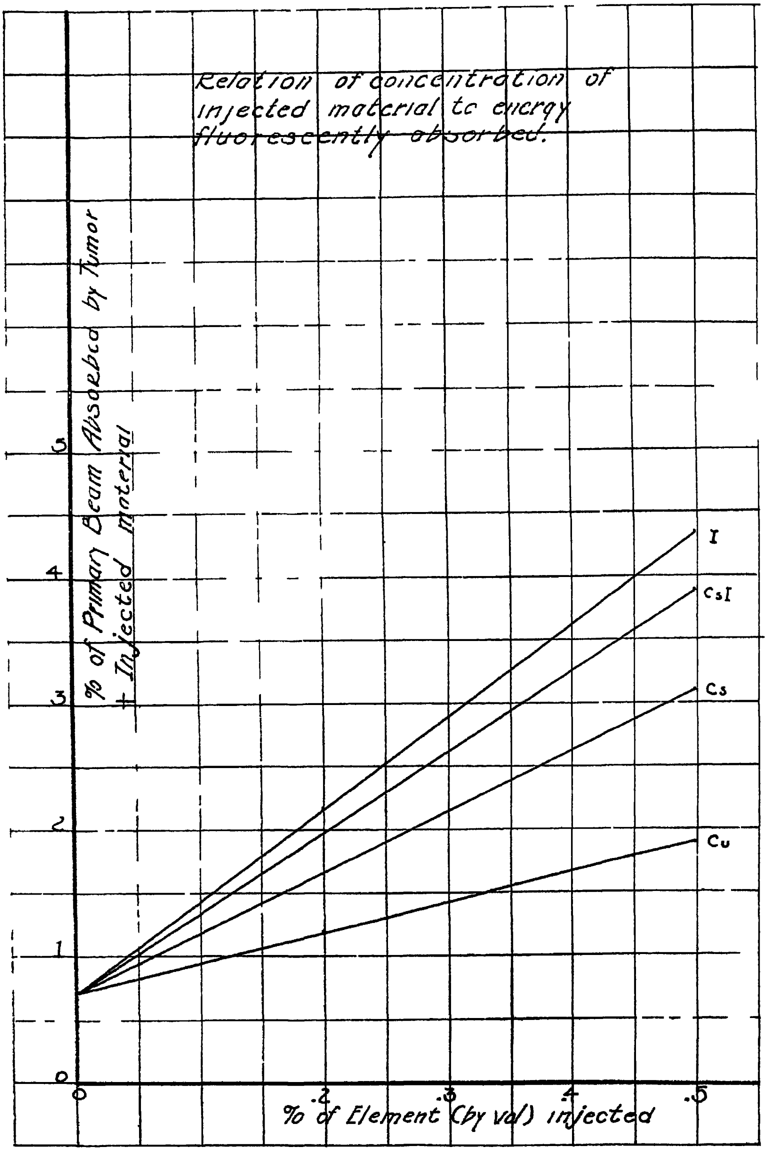

FIGURE IV

Increase in Effective Energy

Summing up, we see that the use of injections of elements in connection with proper X-ray treatments produces in every cell small electron emitters which act also as radiators of X-rays. The effect of these injections may be readily observed by referring to Figure IV (page 20), in which curves for some specific examples are given. The calculations are for a tumor 5 cm. thick treated with radiations of 0.1 A°

Without the injection 0.73% of the total energy incident at the surface of the body is absorbed by the tumor, whereas, with an injection of copper of 0.2% by volume of tumor, the energy absorbed by the tumor mass is 1.4%. The increase due to the injection is 100%.

These values are calculated for the K absorption band of copper. As stated before, the energy absorbed by the copper is not lost. Much of it is given up in such a form that it is easily absorbed by the tumor. With 0.2% of bismuth, the total increase in energy absorbed by the tumor mass is 1370% for a radiation of 0.1 A°. The K characteristic radiation from bismuth, however, is only slightly absorbable by the tumor; therefore it would be very ineffective.

Curves for caesium, iodine, and caesium iodide are given in Figure IV (page 20).

Treatments as outlined in the foregoing paragraphs can be successful only when the proper exciting radiations are used. It is not known what percentage of the energy fluorescently absorbed is changed to characteristic X-rays and what percentage to photo-electrons.

If photo-electrons represent the greater part of the energy derived from fluorescent absorptions, the excitation of the K radiation of bismuth will be more effective than the L, as may be seen from the quantum expression—

$$\text{(3)} \qquad \mathrm{nh}\nu = \mathrm{I}_0 \frac{\tau}{\mu} (1 - e^{-\mu l})$$

in which n is the number of electrons emitted, h a constant, ν the frequency, I_0 the incident energy, and μ and τ the total absorption, and fluorescent absorption coefficients respectively or—

$$\text{(4)} \qquad \mathrm{nh}\nu = \mathrm{I}_0 \frac{\mathrm{C}\,\lambda^3 \quad \mathrm{N}^4}{\mathrm{A}\,\mu} \rho \; (1 - e^{-\mu l})$$

where C has the usual value of 0.00133 for the L absorption band and 0.0136 for the K. Actually C varies with the element in question and for bismuth is considerably less than 0.0136.

Since many compounds are known to fluoresce in the ultra-violet region of the spectrum under the action of X-rays, it is thoroughly possible that with the proper choice of compounds, a combination of corpuscular, atomic, and molecular radiations will be excited.

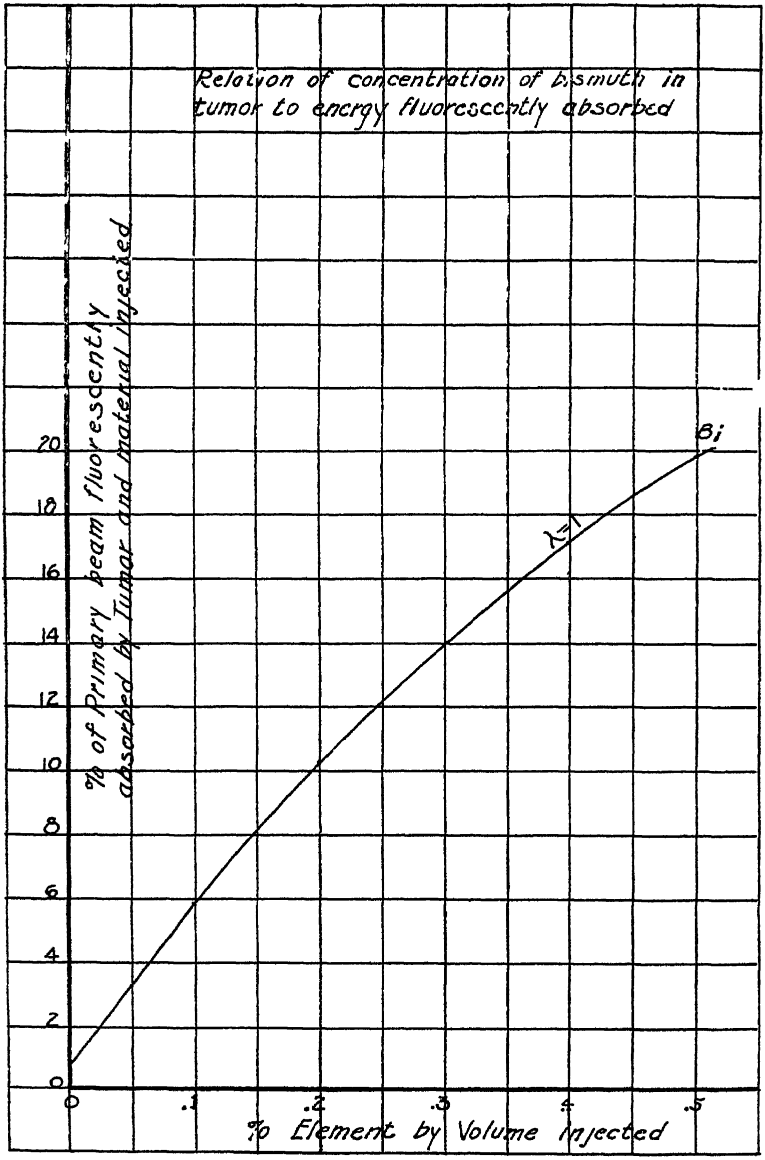

FIGURE V

Caesium chloride, calcium tungstate, and sodium bromide are among the compounds which have been found to fluoresce in the ultraviolet region.

IV

Experimental Injections

In the foregoing theoretical considerations, it has been shown that an increased effect should be obtained by introducing into the body elements of high atomic number, or by introducing substances capable of fluorescence in the ultra-violet region of the spectrum, when excited by X-rays. There is abundant experimental evidence which may be interpreted as showing this action.

Anderson[1] claims to have intensified the action of X-radiation upon cancer by an oatmeal and high salt diet. Perrine[2] in examining the fluorescence of a number of substances found that sodium chloride fluoresced in the mid-ultra-violet region when excited by X-rays. Poos[3] found that when the blood of the frog was replaced by Ringer's solution, the effect of a measured quantity of X-radiation was much less. The greater resistance of tumor tissue in vitro also suggests the possibility that characteristic radiation from the iron of the haemoglobin molecule may play some part in enhancing the effect of X-rays upon the tumor in vivo. The increased destruction of tumor lying close to bone when treated with penetrating X-rays tends to show that characteristic radiations of relatively long wave-length or β-particles are very active in cell destruction.

Calcium Phosphotungstate.

The work of Peacock and Whitby[4] showed that the excitation of bactericidal fluorescence by X-rays is possible. They were able to reduce the number of surviving organisms to as low as 0.7% after irradiation. Their experiments further demonstrated that when a mixture of organisms and calcium phosphotungstate were injected into an animal and irradiated that the resulting lesion was very slight and insignificant as compared with those that were not irradiated. The experiments of Peacock and Whitby, using calcium phosphotungstate, show, however, that the precipitating action of this salt on proteins prevents its use intravenously.

In the selection of a substance to be used in treatment of tumors, the following points must be considered: (1) Low toxicity. (2) Solubility in water. (3) Capacity for fluorescence in ultra-violet region or emission of characteristic radiation of suitable wave-length. (4) High coefficient of absorption for X-rays. (5) Selective absorption of the substance by tumor tissue.

---

[1] Anderson, Ernst, Strahlentherapie, XVIII, 865-870 (1924).
[2] Perrine J O Physical Review, II, 48 (1923)
[3] Poos, Fritz Strahlentherapie, XVIII, 369 374 (1924).
[4] Peacock, P. R. and Whitby, L. H., British J Exp. Path., 8, 43, 49 (1927)

Caesium Iodide.

The work of Perrine had demonstrated that, of all the alkali earth chlorides, caesium chloride gave the most intense fluorescence when excited by X-rays. Von den Velden[1] found tumor cells to have a selective affinity for iodine. Weil[2] showed that necrotic areas of tumors contain more iodine than other tissues of the body following intravenous injection. These factors, together with the fact that the atomic numbers of iodine and caesium are 53 and 55 respectively, led us to the choice of caesium iodide as the substance most likely to meet all the requirements.

The characteristic radiations of caesium and iodine are very closely similar in wave-length. No data is available to compare the intensities of characteristic radiations from each of these elements. The following table prepared from "Siegbahn Spectroscopy of X-Rays" shows the wave-lengths of the various spectral lines of caesium and iodine for the K and L series:

TABLE I

		α_1	α_2	β_1	β_2	γ_1	γ_2
Cs	K	[illegible] cm	[illegible] cm	[illegible] cm	....	....	....
	L	2886	2896	2678	$2506 \cdot 10^{-11}_{cm}$	$2343 \cdot 10^{-11}_{cm}$	$2232 \cdot 10^{-11}_{cm}$
I	K	437	....	388	375	....	....
	L	3142	3151	2931	2746	2577	....

The toxicity of caesium iodide is greater than any of the other compounds of caesium but this defect is more than compensated by its much higher coefficient of absorption.

Apparatus

The apparatus consisted of a Kelley-Koett high voltage deep therapy X-ray unit (Type G. A., Serial 10) in connection with a water-cooled tungsten target Coolidge X-ray tube as a source of radiations. Voltages ranging from 40 to 250 K. V. were obtainable.

The tumor used throughout these experiments was a rapidly growing mouse sarcoma*. This tumor grows rapidly, has a very high percentage of takes, and shows little if any variation in percentage of takes at different times of the year. It reaches its maximum growth in about twenty-one days, and becomes noticeable about seven days following inoculation. The tumors were used only up to the fifteenth or seventeenth day as, after that period, they became necrotic and the development of transplants from them became more irregular.

---

[1] Von den Velden, Berl. Klin. Wchschr., XLIX. 825 (1912).

[2] Weil, R., Jour. Canc. Research, 1, 95 (1916).

*Tumor furnished through the courtesy of Dr. L. C. Strong, Harvard University.

Lethal Dose of X-Rays for Mouse Sarcoma

The determination of a lethal dose of X-rays for transplantable mouse sarcoma was the next step.

The animals selected for these experiments had tumors twelve to fourteen days old, and about 2 cm. in diameter. The tumors selected were as nearly as possible of the same size. The animals were killed, and the tumors removed aseptically. Each tumor was cut in small, uniform pieces, placed in sterile petri dishes moistened with a few drops of Ringer's solution, and placed in position for exposure.

The radiation was filtered through 1½ mm of aluminum. At intervals of ten minutes the radiation was stopped, a portion of the tumor was removed and injected into a series of mice to determine the exact time required to kill the tumor. The results for X-rays alone are shown in the following table.

TABLE II

Showing lethal dose of X-rays alone for mouse tumors of various ages; and normal variation in suceptibility of different tumors to X-rays alone

Peak K. V.—150 M. A.—20 Distance—19″
Filters—1.5 mm. Al.

Series	Age of Tumor	No. Animals in Group	Length of Exposure	Per Cent Tumor Takes
1	11 days	20	10 min.	95
2	14 "	20	10 "	100
3	14 "	20	10 "	95
4	11 "	20	20 "	95
5	17 "	20	20 "	100
6	17 "	20	20 "	100
7	14 "	20	30 "	80
8	18 "	20	30 "	74
9	17 "	20	40 "	0
10	14 "	20	40 "	0

In addition to showing the length of exposure required to destroy the tumor cells, Table I shows that the resistance of the tumor to X-ray is very constant. There is no variation in susceptibility of tumors on different animals, nor is the age of the tumor a factor to be considered in length of exposure necessary to destroy it.

X-Rays and Caesium Iodide

Having determined the time necessary to destroy the tumor with X-rays alone, we began experiments to determine the effect of injecting caesium iodide and then giving various exposures of X-rays. The caesium iodide was injected intravenously, in 5% solution. Four injections, ½ mg. per gram of body weight, were given at intervals of thirty minutes. One hour after the last injection, the animal was killed; the tumor was removed aseptically and cut in small, uniform pieces about 5 mm. square and exposed to the filtered radiations under exactly the same conditions as with X-rays alone. After ten, twenty, thirty and forty minute periods, portions were removed and injected into a series of mice to determine the viability of the tumor.

Table III illustrates results obtained by use of caesium iodide in conjunction with X-ray exposure identical with that listed in Table II.

TABLE III

Caesium Iodide (2 mg. per gm. body wt.) and X-rays.

Series	Age of Tumor	No. Animals in Group	Length of Exposure	Percentage Tumor Takes
1	14 days	20	10 min.	88
2	14 "	20	20 "	12
3	14 "	20	30 "	0
4	14 "	20	40 "	0
5	14 "	20	10 "	88
6	14 "	20	20 "	25
7	14 "	20	30 "	0
8	14 "	20	40 "	0
9 (control)	14 "	20	0 "	100

Figure VI (page 27) shows the effect of X-rays under similar conditions, with and without caesium iodide injections. The increased destructive action of X-rays on tumor tissue containing caesium iodide is quite pronounced. The mechanism of this action may be the emission of relatively long wave-length, characteristic X-radia-

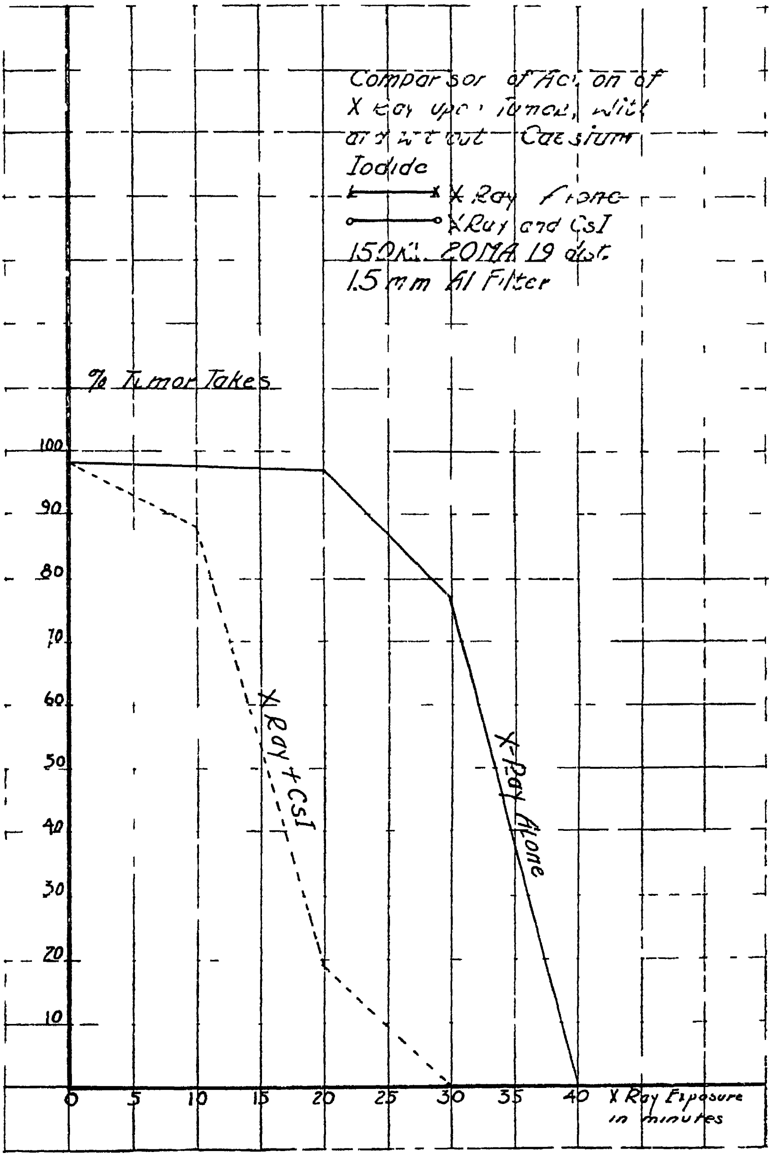

FIGURE VI

tions, of fluorescent radiations, or the emission of photo-electrons and their absorption by the surrounding tissue.

The possibility that the action might be due to decomposition of the salt and liberation of iodine has been investigated. No decomposition whatever has been observed in the case of this salt in solution for periods up to fifty minutes, with the tube operated under the same conditions as used in all other experiments. Reference to Table III, series 9, shows that the presence of caesium iodide alone has no effect upon the percentage of tumors developing after inoculation.

V

SUMMARY

1. The energy absorbed and changed to fluorescent radiations and photo-electrons is probably the part of the absorbed energy which is effective in producing a cure.

2. For every depth and thickness of tumor, there is an optimum band of wave-lengths within which treatments should be given.

3. Only a limited number of possible combinations of elements with radiations of specific wave-length will in general produce the desired results.

4 The lethal dose of X-rays has been determined for a transplantable mouse sarcoma at 150 peak K. V , 20 M. A., 19″ skin target distance.

5. The dose of X-rays necessary to kill a given tumor is independent of the age of the tumor and is the same for all mice.

6. The action of X-rays upon mouse sarcoma in vitro is very much increased by the presence of caesium iodide in the tumor.

7. This action is to be ascribed to one or all of the following factors:

(a) Characteristic radiation of relatively long wave-length.

(b) Fluorescent radiation in ultra-violet region when excited by X-rays.

(c) Emission and subsequent absorption of photo-electrons.

CALCULATIONS

Part I

In Diagram A (page 12), let

I_o=the energy of the incident radiation.
I_a=the energy at a depth d_1.
I_b=the energy at a depth $d_1 + d_2$

From the well-known law of absorption—

$I_a = I_o e^{-\mu_1 d_1} = I_o' e^{-d_1(\tau_1 + \sigma_1)}$ where e is the base of natural logarithms.

$$I_b = I_a e^{-\mu_2 d_2} = I_o e^{-d_2(\tau_2 + \sigma_2)} \cdot e^{-d_1(\tau_1 + \sigma_1)}$$

where μ, τ and σ are the total absorption coefficient, fluorescent absorption coefficient and scattering coefficient respectively; the subscripts 1 and 2 referring to layers d_1 and d_2 respectively.

The percentage of I_a absorbed in d_2 is given by—

$$\frac{I_a - I_b}{I_a} = (1 - e^{-\mu_2 l_2})$$

and the percentage of I_o absorbed in d_2 is given by

(Equation 3) $\alpha_2 = (1 - e^{-\mu_2 d_2})\, e^{-\mu_1 l_1}$

The wave-length at which maximum absorption (total) of energy occurs may be obtained from the equation—

$$\frac{d\,\alpha_2}{d\,\lambda} = 0$$

where λ is the wave-length of the incident radiation.

Let—

$$b_1 = \sigma_1 d_1 \qquad a_1 = \frac{C_1 N_1^4 \rho_1 d_1}{A_1}$$

$$b_2 = \sigma_2 d_2 \qquad a_2 = \frac{C_2 N_2^4 \rho_2 d_2}{A_2}$$

$$\mu_1 = \tau_1 + \sigma_1 \qquad \mu_2 = \tau_2 + \sigma_2$$

$$\frac{d\alpha_2}{d\lambda} = 0 = e^{-a_1 \lambda^3 - b_1}(-3a_1 \lambda^2)$$

$$- e^{-[\lambda^3(a_1 + a_2) + (b_1 + b_2)]} \cdot [-3(a_1 + a_2)\lambda^2]$$

from which

$$\text{(Equation 4)} \quad \lambda = \left[\frac{\log \left(1 + \frac{C_2 N_2^4 \rho_2 d_2 A_1}{C_1 N_1^4 \rho_1 d_1 A_2} \right) - \sigma_2 d_2}{\frac{C_2 N_2^4 \rho_2 d_2}{A_2}} \right]$$

Part II

The energy scattered in d_2 (the tumor) may be determined in the following manner:

Let I_σ = the amount of energy scattered in a medium at any depth x.

I_p = the energy at this depth x, then—

$$I_p = I_o e^{-\mu x}$$

$$dI_\sigma = \sigma I_p dx$$

$$I_\sigma = \int_0^x \sigma I_o e^{-\mu x} dx$$

$$I_\sigma = \left[\frac{\sigma I_o e^{-\mu x}}{-\mu} \right]_0^x$$

$$= - \frac{\sigma I_o (1 - e^{-\mu x})}{\mu}$$

If I_s = the energy scattered in d_2, and μ_2, σ_2, τ_2, μ_1, σ_1 and τ_1 are the same as in Part I, we may write—

$$I_s = \frac{\sigma_2 I_1 (1 - e^{-\mu_2 d_2})}{\mu_2}$$

$$= \frac{\sigma_2 I_o e^{-\mu_1 d_1} (1 - e^{-\mu_2 d_2})}{\mu_2}$$

If β_2 = the percentage of I_o scattered in d_2 we have

$$\text{(Equation 5)} \quad \frac{I_s}{I_o} = \frac{\sigma_2}{\mu_2} (1 - e^{-\mu_2 d_2}) e^{-\mu_1 d_1}$$

$$= \frac{\sigma_2 \alpha_2}{\mu_2}$$

The wave-length at which β_2 is a maximum may be obtained from the equation—

(Equation 6) $$\frac{d\beta_2}{d\lambda} = 0 = \frac{d}{d\lambda}\left(\frac{\sigma_2}{\mu_2} \cdot \alpha_2\right)$$

which mav be solved for λ.

Part III

In a manner similar to that employed in Part II, it can be shown that—

$$I_f = \frac{\tau_2\ I_o\ e^{-\mu_1 d_1}\ (1 - e^{-\mu_2 d_2})}{\mu_2}$$

where I_f = the energy fluorescently absorbed in d_2.

γ_2, the percentage of the total primary beam fluorescently absorbed in d_2 is given by—

(Equation 7) $$\gamma_2 = \frac{I_f}{I_o} = \frac{\tau_2}{\mu_2}\ (1 - e^{-\mu_2\ 2})\ e^{-\mu_1 d_1}$$

The wave-length at which γ_2 is a maximum may be obtained from the equation—

(Equation 8)

$$\frac{d\gamma_2}{d\lambda} = 0 = e^{-(a_2\lambda^3+b_2)} \cdot \left[-b_2+(a_1+a_2)\lambda^3(a_2\lambda^3+b_2) \right. \\ \left. + b_2 - a_1\lambda^3(a_2\lambda^3+b_2) \right]$$

where $a_1 = \dfrac{C_1\ N_1^4\ \rho_1\ d_1}{A_1}$ $\qquad b_1 = \sigma_1\ d_1$

$a_2 = \dfrac{C_2\ N_2^4\ \rho_2\ d_2}{A_2}$ $\qquad b_2 = \sigma_2\ d_2$

By substituting the above values for a_1, a_2, b_1 and b_2 the value of λ for γ_2 to be a maximum may be found.

The writers are deeply indebted to the Kelley-Koett X-Ray Manufacturing Company of Covington, Kentucky, for their generosity and hearty cooperation, without which these experiments could not have been conducted.

A COMPARATIVE STUDY OF TOXICITIES OF CERTAIN GROUPS OF CHEMICAL SUBSTANCES USED IN CANCER TREATMENT

By LEONORA NEUFFER and ROBERT J. NORRIS*

In a few cases, experimenters[1] have mentioned the irradiation of cancerous tissue in combination with chemo-therapy (lead being the substance administered) but to the best of our knowledge no one has approached the problem from the standpoint of exciting secondary radiations of such a character as would produce the desired biological effects.

From the physicist's viewpoint a greater efficiency in the treatment of cancer with X-rays may be obtained by increasing the concentration of elements of high atomic numbers in the tumor over that in the normal tissue.

If hard X-rays (short wave-lengths) are then used, the absorption by the superficial tissue will be relatively small while the absorption by the tumor mass (tumor and injected material) will be relatively large.

The energy fluorescently absorbed by the injected materials will be partially converted into soft X-rays and photo electrons, both of which are very active biologically.

The reason for choice of elements of high atomic numbers may be seen by referring to the following equation for the absorption of X-rays:

$$I_a = I_o \, e^{-(K N^4 \lambda^3 + \sigma) d}$$

where I_a = intensity at depth d.

I_o = intensity of incident radiation.

N = atomic number.

λ = wave-length.

e = base of natural logarithms.

From the physiological viewpoint, the substance used as a source of secondary radiations should be non-toxic as well as soluble in water or dilute alkali.

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*Dr. Neuffer directed the chemical phases and Mr. Norris the biological phases of this experiment.

[1] Ullman, Radiology. June, 1927. Bell, Lancet, 207, 267 (1924).

The present paper gives an account of the substances, intravenously injected into white mice, and toxicity data obtained when the animals were observed daily for a period of two weeks Our selection of substances was based on several considerations in addition to the properties listed above. We desired to make a comparison among elements in the same group in the periodic table as well as among elements in different groups, and also to show differences in toxicity due to certain alterations in chemical constitution.

I

Groups of Substances

Commercial Medicinals

Before undertaking the laboratory preparation of desirable substances, a large number of commercially obtainable medicinals, containing high atomic weight elements, was used These included aristol, sajodin, dermatol, bismuth B-naphthol, bismuth subsalicylate, mercurochrome, iodival, arrhenal, airol, soziodol, iodipin, atoxyl, arsacetin, mercury succinimide, neosalvarsan, silver salvarsan, mercurosal, and mercodel. Such data as were obtainable are recorded on the charts (pages 38-45).

Rarer Elements

The solutions of nitrates and chlorides of some of the rarer elements, vanadium, thallium, cerium, neodymium, titanium, zirconium, lanthanum, erbium, and yttrium, were found unsatisfactory for injection because of their acid character. Our plan is to use these elements, if possible, in salts which do not show an acid reaction.

Bismuth Compounds

Following the suggestion of Supinewski and Adams[1] with reference to the possible value in chemotherapy of certain bismuth organic compounds, and considering their statement that the possibility of obtaining water-soluble carbon-bismuth compounds had been kept specially in mind in their work, we prepared some of these substances.

Tri-o-tolyl- and tri-p-tolyl-bismuth and their dichlorides were made according to the methods of Pfeiffer and Pietzsch[2], Challenger[3], and Supinewski and Adams[1]. The substances were found to be insoluble in water or dilute alkali and therefore could not be used for intravenous injection.

---

[1] Supinewski and Adams. J Am Chem Soc, 48, 507 (1926).
[2] Pfeiffer and Pietzsch, Ber, 37, 4622 (1904).
[3] Challenger, Proc. Chem Soc, 29, 76 (1913).

Harden and Dunning[1] have mentioned the probable pharmacological value of bismuth thioglycolamide. We prepared this and found it to be much too toxic for our purpose.

Iodides of Alkali Metals

A comparison of the toxicities of the iodides of the alkali metals was made. The iodides of lithium, rhubidium, and caesium were prepared from the sulphates by treatment with barium iodide.

Caesium Compounds

The element, caesium, was injected in the forms of chloride, nitrate, iodide, and trichloracetate. The last consists of colorless needles, easily soluble in water and infusible. It was prepared by the action of caesium carbonate on trichloracetic acid.

Colloids

The general impression left by investigators seems to be that metals are less toxic in colloidal than in the ionic state. There exists some difference of opinion in this matter, however. With reference to colloidal arsenic, antimony, and silver, Clark[2] states in his summary that pharmacological tests fail to show any marked differences between the action of colloidal preparations tested and the action of the same substances in true solution. Bell[3], on the other hand, in describing his work on the injection of colloidal lead intravenously in the treatment of cancer, has stated that colloidal lead is less toxic than ionic lead, and that lead has a specific action on cancer tissue.

With a view to finding a substance still more satisfactory than lead, we have investigated the toxicities of other colloids including iodine, prepared according to Chandler and Miller[4], gold prepared by the Zsigmondy method, selenium resulting from the reduction of selenium dioxide by hydrazine hydrate, and copper obtained electrolytically by the Bredig method. In this last preparation, a direct current of 8 amperes with a potential of 108 volts was used. Copper electrodes made of thin copper sheet cut in strips about ¼ inch wide were dipped into a solution containing conductivity water, 0.027% of calcium chloride and 0.4% gelatin.* The use of colloidal selenium in cancer treatment has been reported by Walker[5]. It was found by him to be non-toxic.

---

[1] Harden and Dunning, J. Am. Chem. Soc., 49, 1017 (1927).
[2] Clark, Brit. Med. Jour., Feb. 17, 1923, 273.
[3] Bell, Lancet, 207, 267 (1924).
[4] Chandler and Miller, J. Phys. Chem. 31, 1091 (1927).
*Most of the work on the preparation of colloids has been done by Miss Elizabeth Shelow of this aboratory staff.
[5] Walker, Lancet, 182, 1337 (1912).

Before injection all colloids were carefully dialysed in colloidion bags until the dialyzates showed no test for free ions. Data are given in the charts (pages 38-45) for iodine and selenium. Copper and gold in concentrations used were not lethal in the largest doses possible for injection. Colloidal tellurium, made by the reduction of tellurium dioxide by hydrazine hydrate, and colloidal silver, prepared by the method of Gubtier and Hofmeier[1], and other colloids will be used.

If colloids are to be of real value therapeutically, no doubt special investigation will be necessary in the future from the standpoint of obtaining colloids in much more highly concentrated solutions than those commonly known. Stabilization will also need further study. We have attempted to obtain some comparative data between colloidal elements and the same elements in chemical combination. For gold in combination, sodium aurothiosulphate was used. Its therapeutic properties are mentioned by Brown[2] in an article describing its preparation. Mercury, iodine, selenium, and tellurium have been used in the form of phthalein derivatives.

Phthaleins

The most promising part of this toxicity study has been the use of phthaleins* and their derivatives. The substances show relatively low toxicities, are soluble in dilute alkalies, and seem to be particularly adapted for use in investigation of selective absorption by cancer tissues which is to be undertaken in this laboratory. Phenolphthalein, fluorescein, and their bromine, iodine, mercury, selenium, and tellurium derivatives were used with a view to determining comparative effects of certain high atomic weight elements when introduced into the simple phthalein molecules. Use of selenium eosin in cancer treatment has been reported by Walker[3], Wassermann[4], and others[5].

The dibrom-, diiodo-, tetrabrom-, and tatraiodo-derivatives of phenolphthalein and of fluorescein compounds in which the halogen is in the phenolic rings were prepared by direct halogenation. Reactions required from eight to sixteen hours, some taking place at room temperature and some requiring heat. Recorded methods[6] were used for the tetra- compounds. The di- compounds were obtained similarly by limiting the amount of halogen. Diiodophenolphthalein is a yellowish white solid, very slightly soluble in water, soluble in dilute alkalies and reprecipitated by dilute acids. The substance decomposes before melting. Octaiodofluorescein, having four iodines in the phthalic anhydride nucleus and two in each of the phenolic nuclei, was made, as described by Pratt and Coleman.[1] by halogenating phthalic anhydride, combining with fluorescein, and halogenating again.

---

[1] Gubtier and Hofmeier, Zeit. Anorg. Chem., 45, 77 (1905).

[2] Brown, J. Am. Chem. Soc., 49, 958 (1927).

*Many of the substances used in this work were prepared with the valuable assistance of Abe Gelperin, a student at the University of Cincinnati.

[3] Walker, Lancet, 182, 1337 (1912)

[4] Wassermann, Zeit. fur Hygiene, 51 (1905).

[5] Fortschritte der Theerfarben Fabrikation, Vol. 12, 806-809.

[6] Barrowcliffe, Organic Medicinal Chemicals, p. 182. Orndorff and Mahood, J. Am. Chem. Soc., 40, 937 (1918). Gomburg and Tabern, J. Ind. and Engin. Chem., 14, 1116 (1922).

Since mercurochrome, which is dibrommercurifluorescein, gave promising results when injected intravenously, it was believed that an iodine analogue might be much more satisfactory. Diiodomercuriphenolphthalein was made by a method similar to that used by White[2] for mercurochrome. The product is a white amorphous solid, soluble in glacial acetic acid from which it is precipitated by water, soluble in dilute alkalies, and precipitated by dilute acids. The substance decomposes before melting.

The literature concerning the preparation of selenium and tellurium phthaleins is meager. Methods of preparation are given in a series of German patents[3]. With reference to chemical constitution, it is indicated in one of these patents that selenium or tellurium takes the place of the oxygen which joins the two resorcinol rings in fluorescein compounds. We have at least found that the proportion of selenium justifies this conclusion. To prepare selenium eosin, fluorescein erythrosin, and tellurium erythrosin, we used the method described in the patents by Jager for making selenium dichlorofluorescein. We found, however, that much longer heating was required than was called for in the patent.

Selenium and tellurium erythrosin are described here for the first time, the former being a dark reddish brown powder, and the latter dull red. All of the selenium and tellurium phthaleins are little soluble in water and readily dissolved by dilute alkalies. They are precipitated by dilute acids. In analyses, sulphide precipitations of selenium and tellurium were used.[4]

For further comparisons, selenium, tellurium, iodine, bromine, and mercury derivaties of phenolsulphonphthalein, cresolsuphonphthalein, and resorcinolsulphonphthalein are being prepared.

II

CHARTS

In the toxicity study, data for which are given in the accompanying charts (pages 38–45), phthalein derivatives were used in dilute alkline solution. All other substances were in water solution. Of necessity, concentrations of the various solutions differed.

---

[1] Pratt and Coleman, J. Am. Chem Soc., 40, 248 (1918).

[2] White, J Am Chem. Soc, 42, 2364 (1920)

[3] Fortschritte der Theerfarben Fabrikation, Vol. 21, 808-809.

[4] Scott, Methods of Analysis, Vol. 1, 420.

The purpose of the charts is to facilitate comparisons of toxicities when certain alterations were made in chemical constitution of substances. Each chart consists of two parts. The figures in Part I represent the maximum non lethal dose of *substance*, injected intravenously in milligrams per gram of body weight of white mice. The figures in Part II represent the weight of the supposedly active element introduced in each case.

It is the plan of this laboratory to study the effect of irradiating by X-rays the most promising of the above named substances and others containing elements with high X-ray absorption. A comparative study of toxicities from the standpoints of positions of elements in the periodic table and alterations of chemical constitution will be continued.

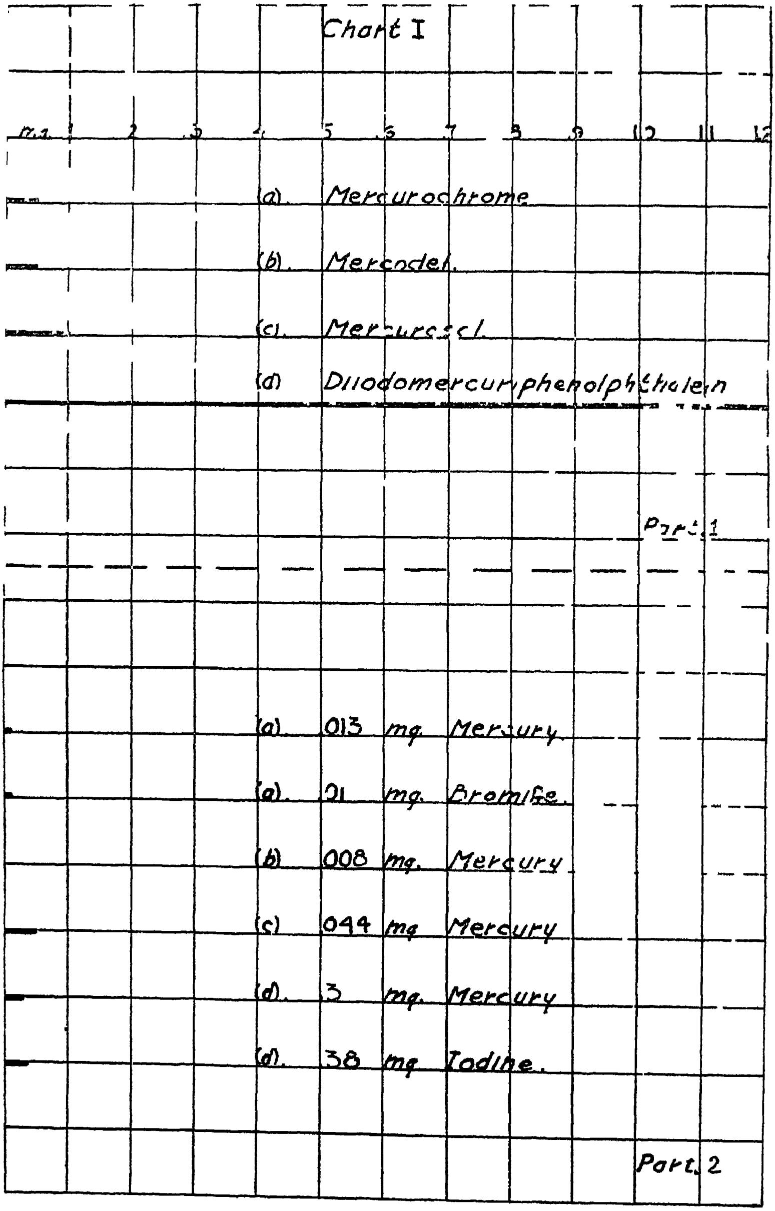
Chart I
m.g. 1 2 3 4 5 6 7 8 9 10 11 12
(a). Mercurochrome
(b). Mercodel.
(c). Mercurosal.
(d) Diiodomercuriphenolphthalein
Part 1
(a). 013 mg. Mercury.
(a). 01 mg. Bromide.
(b) 008 mg. Mercury
(c) 044 mg Mercury
(d). 3 mg. Mercury
(d). 38 mg Iodine.
Part. 2

FIGURE I

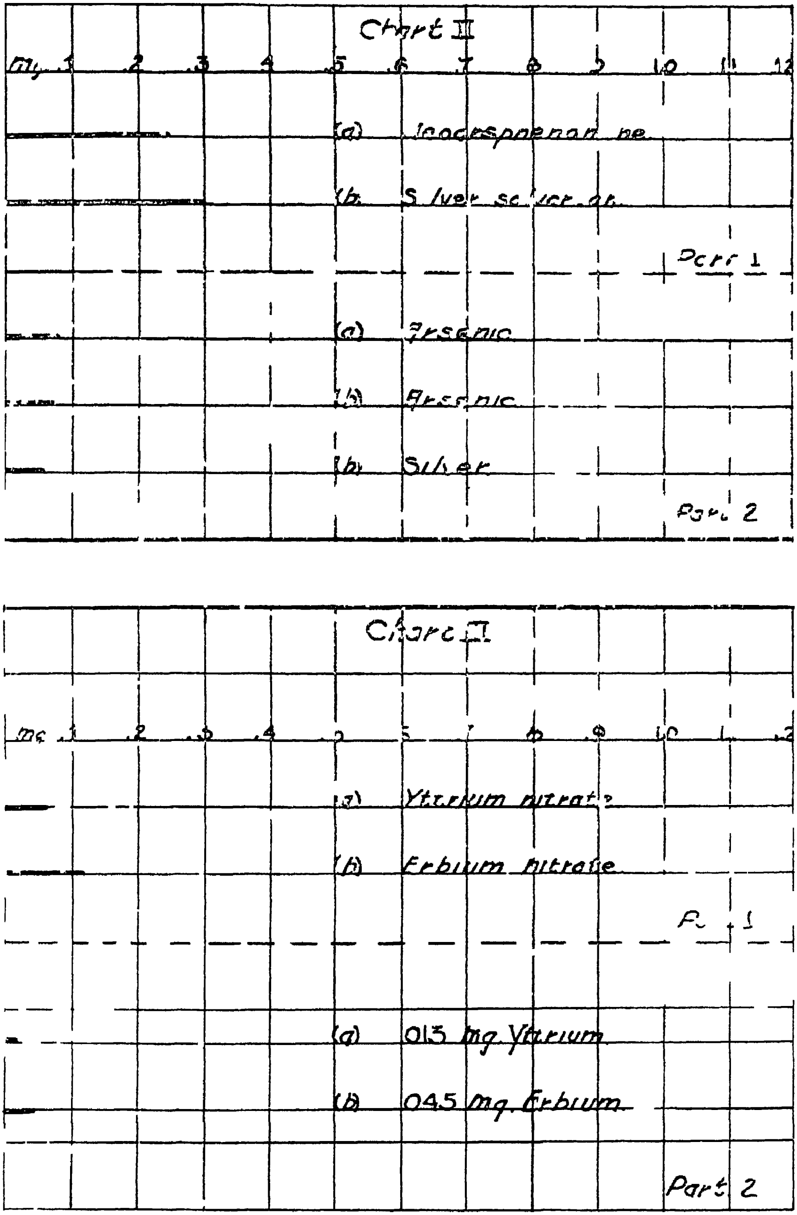
Chart II
mg .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0 1.1 1.2
(a) Neoarsphenamine
(b) Silver salvarsan
Part 1
(a) Arsenic
(b) Arsenic
(b) Silver
Part 2
Chart II
mg .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0 1.1 1.2
(a) Yttrium nitrate
(b) Erbium nitrate
Part 1
(a) 013 mg. Yttrium
(b) 045 mg. Erbium
Part 2

FIGURE II

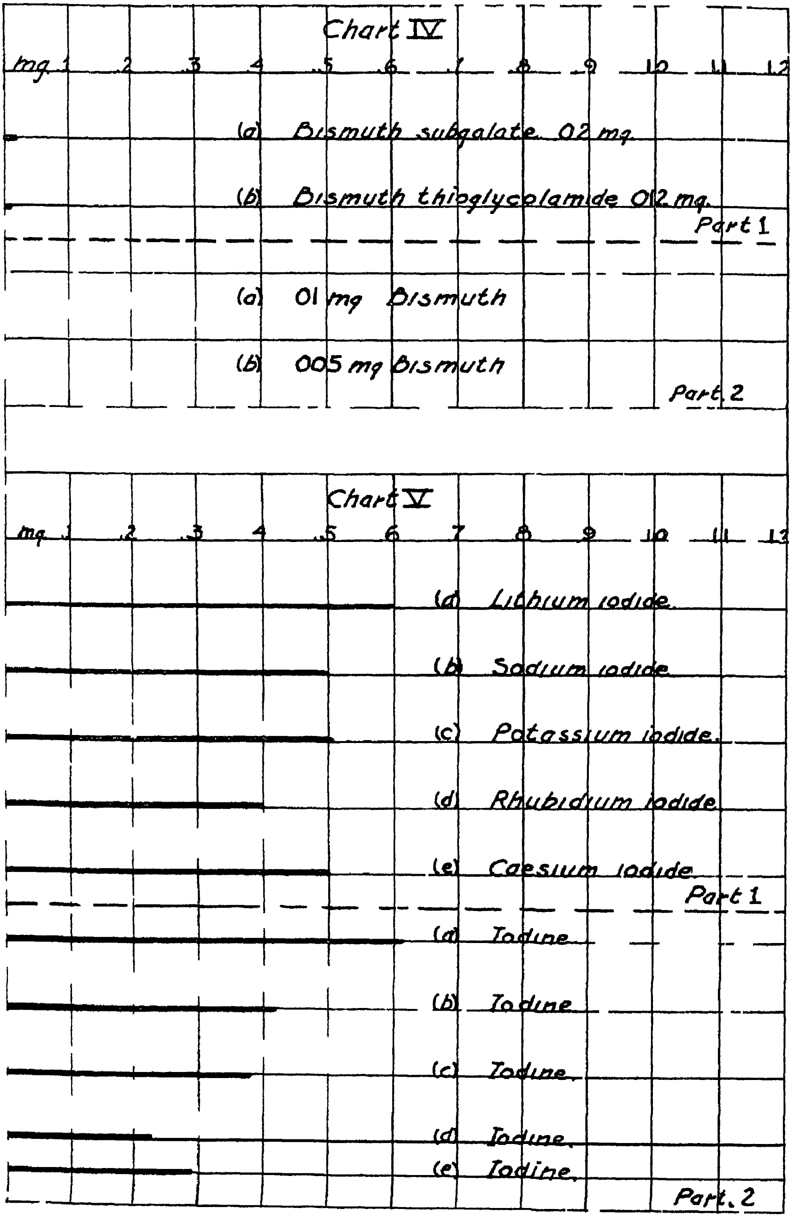
Chart IV
mg. .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0 1.1 1.2
(a) Bismuth subgalate .02 mg.
(b) Bismuth thioglycolamide .012 mg.
Part 1
(a) .01 mg Bismuth
(b) .005 mg Bismuth
Part 2
Chart V
mg. .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0 1.1 1.2
(a) Lithium iodide.
(b) Sodium iodide.
(c) Potassium iodide.
(d) Rhubidium iodide.
(e) Caesium iodide.
Part 1
(a) Iodine.
(b) Iodine.
(c) Iodine.
(d) Iodine.
(e) Iodine.
Part. 2

FIGURE III

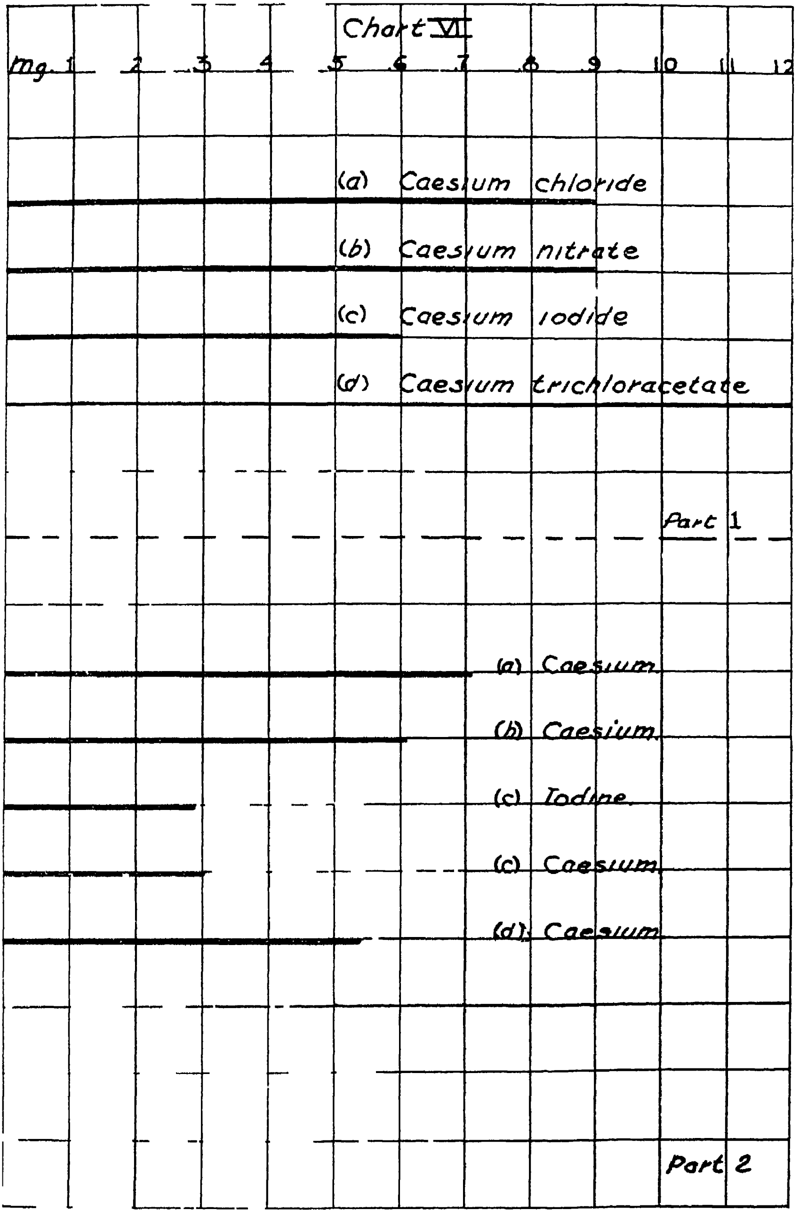
Chart VI
mg. 1 2 3 4 5 6 7 8 9 10 11 12
(a) Caesium chloride
(b) Caesium nitrate
(c) Caesium iodide
(d) Caesium trichloracetate
Part 1
(a) Caesium
(b) Caesium
(c) Iodine
(c) Caesium
(d) Caesium
Part 2

FIGURE IV

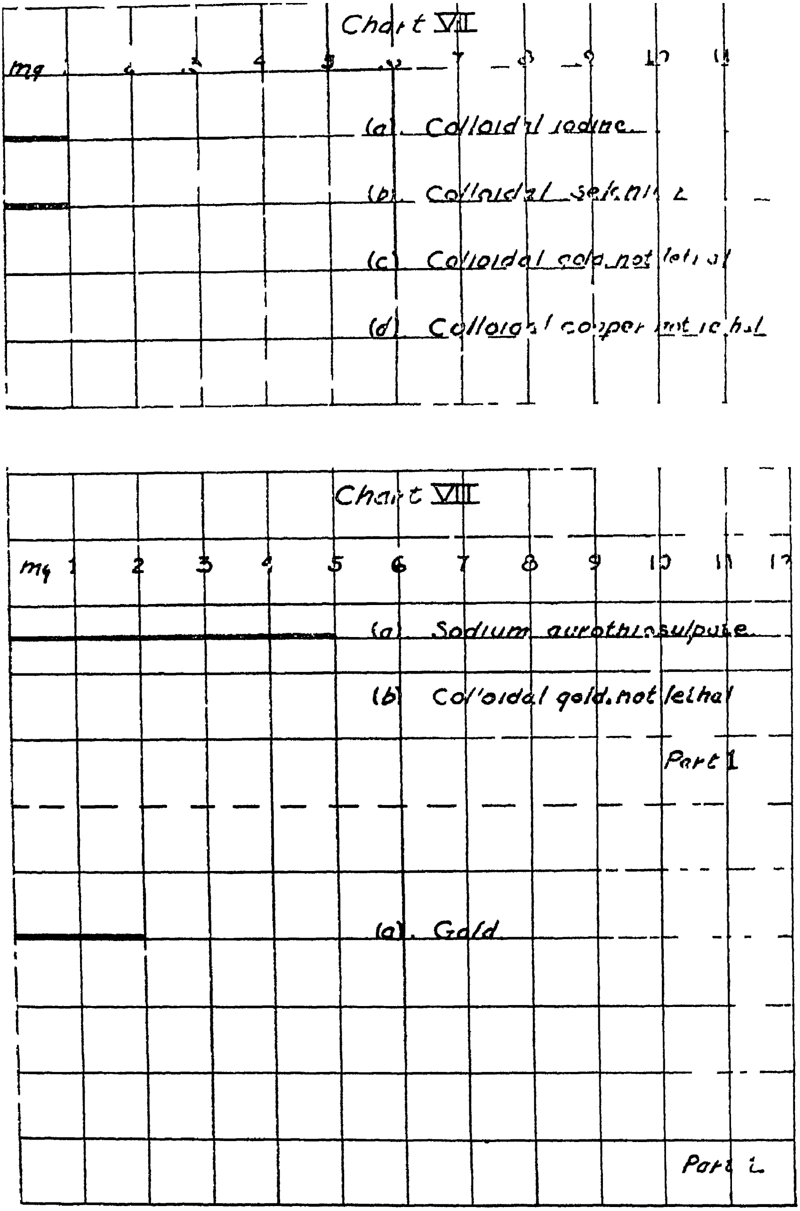
Chart VII
mg 1 2 3 4 5 6 7 8 9 10 11
(a). Colloidal iodine
(b). Colloidal selenium
(c) Colloidal gold not lethal
(d) Colloidal copper not lethal
Chart VIII
mg 1 2 3 4 5 6 7 8 9 10 11 12
(a) Sodium aurothiosulphate
(b) Colloidal gold not lethal
Part 1
(a). Gold
Part 2

FIGURE V

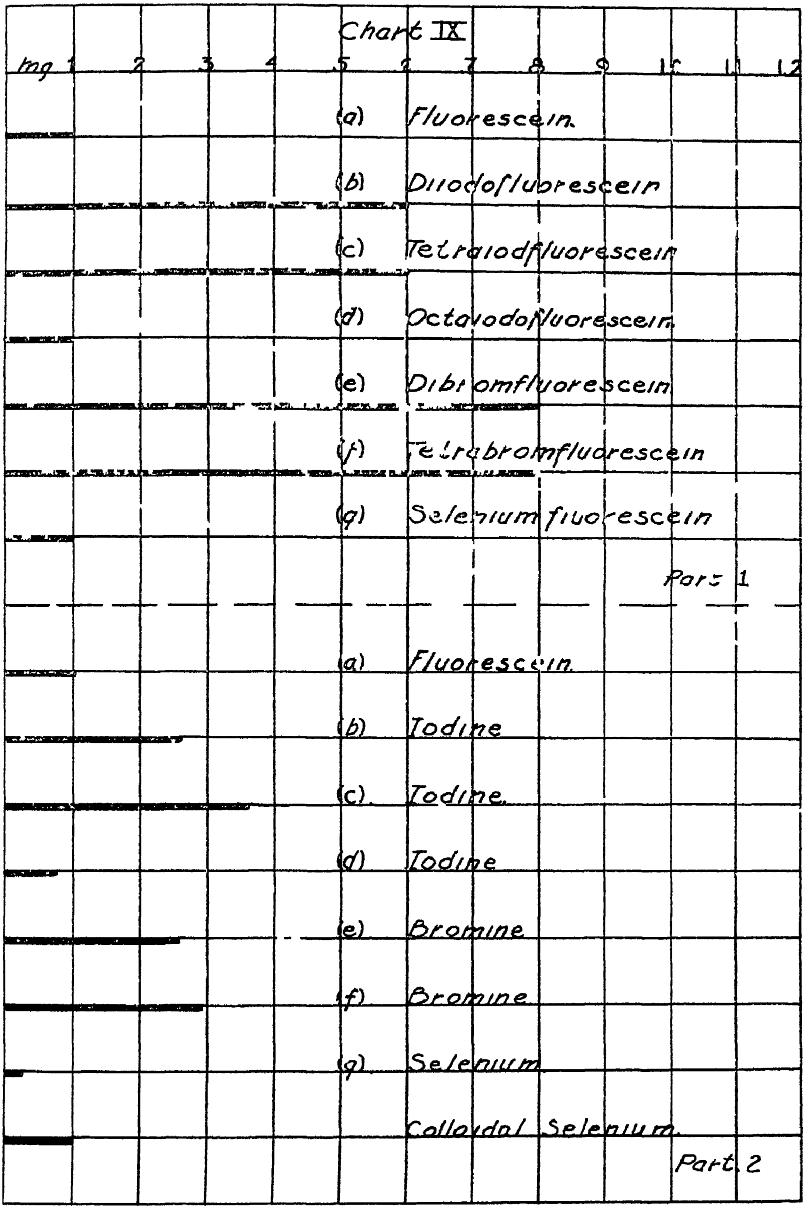
Chart IX
mg 1 2 3 4 5 6 7 8 9 10 11 12
(a) Fluorescein.
(b) Diiodofluorescein
(c) Tetraiodfluorescein
(d) Octaiodofluorescein.
(e) Dibromfluorescein
(f) Tetrabromfluorescein
(g) Selenium fluorescein
Part 1
(a) Fluorescein.
(b) Iodine
(c) Iodine.
(d) Iodine
(e) Bromine.
(f) Bromine.
(g) Selenium
Colloidal Selenium.
Part 2

FIGURE VI

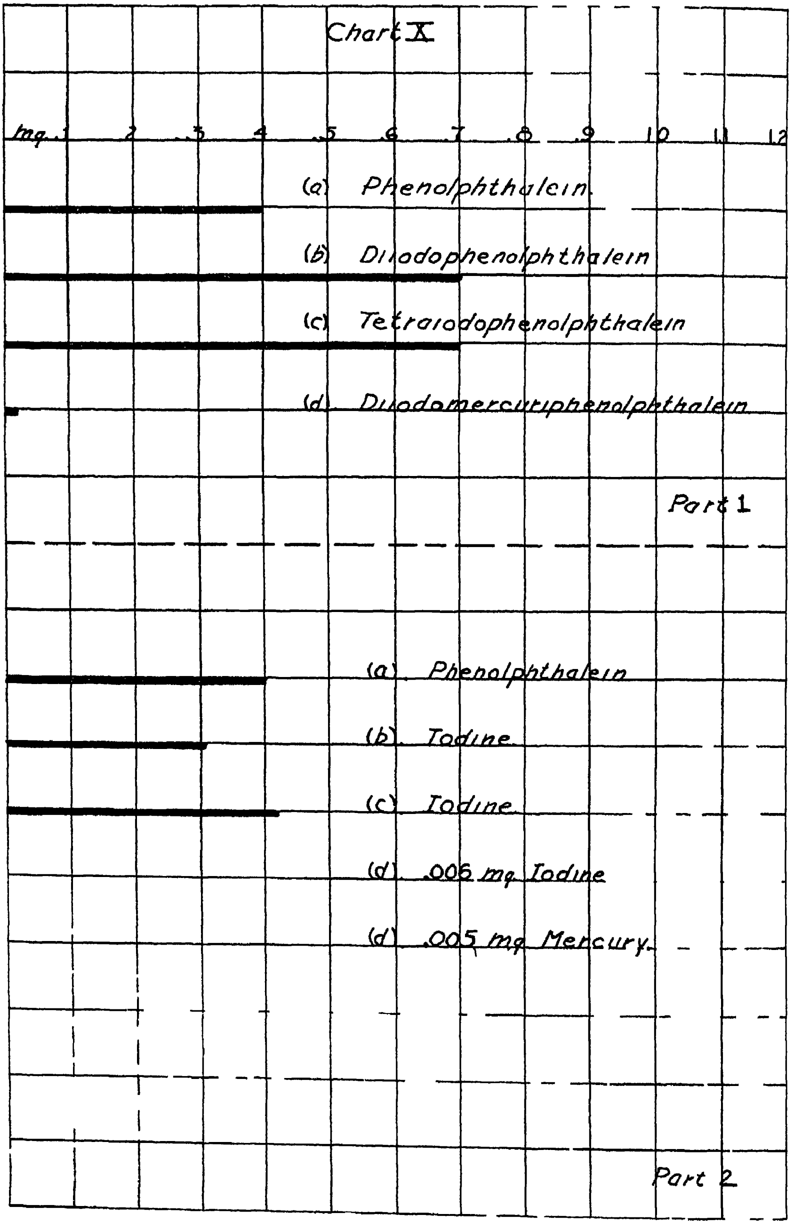
Chart X
mg. .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0 1.1 1.2
(a) Phenolphthalein
(b) Diiodophenolphthalein
(c) Tetraiodophenolphthalein
(d) Diiodomercuriphenolphthalein
Part 1
(a) Phenolphthalein
(b) Iodine
(c) Iodine
(d) .006 mg. Iodine
(d) .005 mg. Mercury
Part 2

FIGURE VII

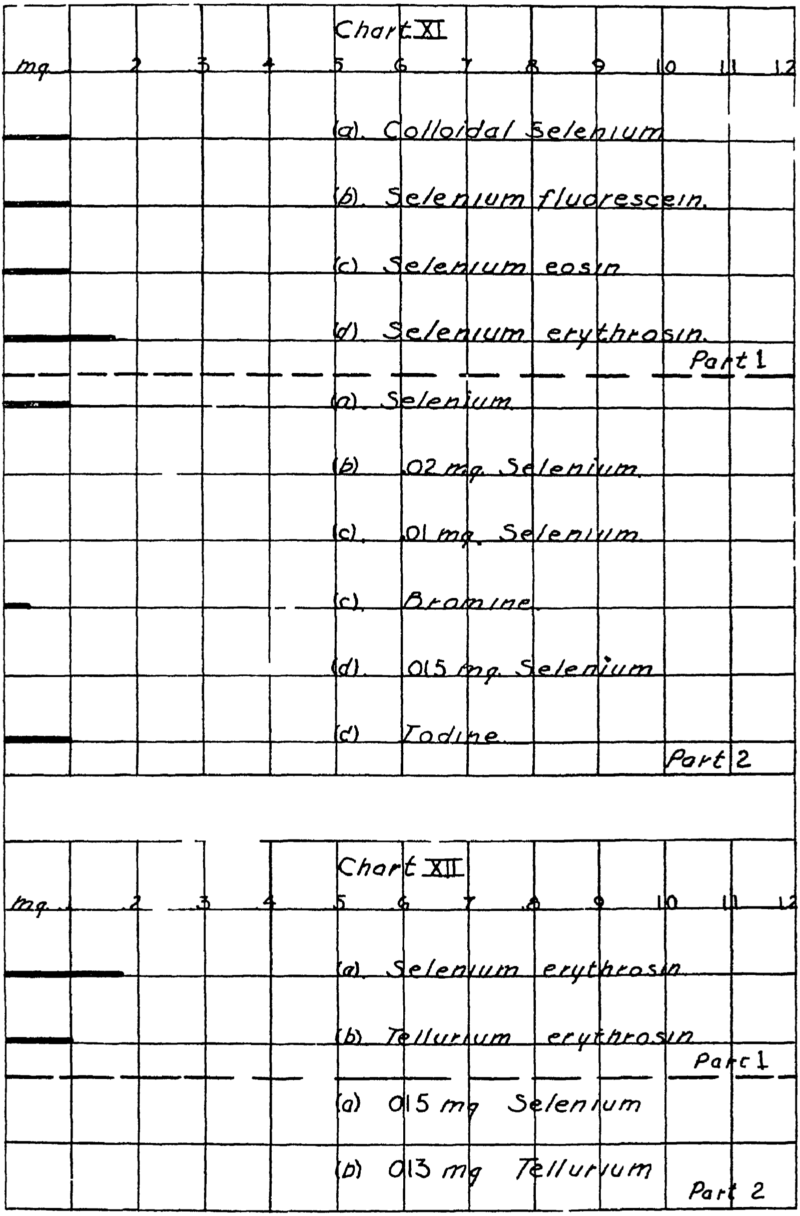
Chart XI
mg. 2 3 4 5 6 7 8 9 10 11 12
(a). Colloidal Selenium
(b). Selenium fluorescein.
(c) Selenium eosin
(d) Selenium erythrosin.
Part 1
(a). Selenium.
(b) .02 mg. Selenium.
(c). .01 mg. Selenium.
(c). Bromine.
(d). .015 mg. Selenium
(d) Iodine.
Part 2
Chart XII
mg. 2 3 4 5 6 7 8 9 10 11 12
(a) Selenium erythrosin.
(b) Tellurium erythrosin
Part 1
(a) .015 mg Selenium
(b) .013 mg Tellurium
Part 2

FIGURE VIII

CRITICAL WAVE-LENGTHS IN KILLING BACTERIA

By George N. Burger

Many investigators have studied the bactericidal action of radiations with the view of determining the relative activities of the various portions of the spectrum. Their results have been quite varied.

Newcomer[1], working with bacillus typhosus found that this organism was killed between 2960 A° and 2100 A°. Browning and Russ[2], using agar surface cultures, a tungsten lamp, and a quartz spectrometer, give 2960 A° to 2100 A°. Cernovodeanu and Henri[3], using a Cooper-Hewitt lamp and bacterial emulsions, found a maximum at about 2800 A°. Mashimo[4], using agar surface cultures and a quartz spectrometer, found the range to be from 2950 A° to 1860 A° with a maximum at 2750 A°. Baynes-Jones[5] and Linder[5] give 3500 A° to 1856 A°.

If it be true that a theory such as the quantum theory[6] is applicable to biology, then, in dealing with the destruction of bacteria by radiations, a definite wave-length at which germicidal action begins should exist. Should such a wave-length exist, the question arises whether it is the same for all bacteria.

With these points in view, the experiments embodied in this paper were undertaken.

I

Filtered Radiations

Filters were prepared, by using varying concentrations of lead acetate in acetic acid. These filters were so designed as to absorb and remove ultra-violet radiations of short wave-length, allowing the longer radiations of the ultra-violet to penetrate. By altering the concentration, portions of the spectrum could be removed as desired. The shortest wave-length to penetrate a given solution was determined by means of a quartz spectrograph[6].

Bacteria to be killed were grown in broth for twenty-four hours and this broth culture was smeared in a thin layer over the surface of sterile nutrient agar in a petri dish. A bent glass rod was used as an applicator to give an even distribution of bacteria without scratching the agar. A piece of cardboard with a small hole in the center was placed over the dish and a quartz cup containing the filter covered the hole. This was then exposed to the lamp at a distance of 30 cm. Plates containing the bacteria were exposed first by keeping the time constant and varying the filter and then, when the proper filter was found, by varying the time.

---

[1] Newcomer, J. Exp Med. 841 (1917).

[2] Browning and Russ, J S. C. I. 1146 (1917).

[3] Cernovodeanu and Henri, Compt. rend , 150, 52 (1910).

[4] Mashimo, Memo, Col. I. Sci. Kyot. 4, 1 to 11 (1919), J. S. C. I., 161, A (1921).

[5] Baynes-Jones, Linder, Bull Johns-Hopkins Hosp. 34, 11, Jan. (1923).

[6] Quantum Theory in Biology, Schneider and Sperti, Series 4, Paper 1, University of Cincinnati, (1926).

Organisms of the colon-typhoid group were killed regularly at 2750 A°. With the next filter, 2800 A° the effect varied with time; in five minutes no effect was noticed; in two hours there was a slight inhibition of bacterial growth.

Bacillus dysenteria (Shiga) was killed at 2800 A° in five minutes, while no effect was found with the 2880 A° filter in an equal interval of time. In two hours there was a slight inhibition with the 2880 A° filter.

It was obvious that the accuracy required in this investigation was far greater than could be afforded by filters.

II

Monochromatic Radiations

In order to obtain an increased accuracy, it was decided to attempt a reproduction of a known spectrum, such as that of the quartz mercury arc, upon bacterial coated agar. Sterile microscopic slides were placed in petri dishes and covered with nutrient agar, each slide receiving a coating of agar about 2 mm. in thickness. These were allowed to stand until the medium was well fixed to the slides. The agar was carefully trimmed off on the sides and at one end to insure safe handling. One drop of a twenty-four hour broth culture of bacteria was then placed on the agar of each slide. A sterile glass rod was carefully passed over the surface to spread out the bacteria thoroughly. After the bacteria had settled completely, a slide was fitted in a frame of formica and placed in the spectrograph. Slides were exposed for varying lengths of time, returned to the petri dishes and incubated for approximately twenty-four hours. Many obstacles were encountered in obtaining good prints, but some good ones were obtained in practically every instance. When the spectral lines were clear, an abrupt end to the bactericidal action was observed in the near ultra-violet portion of the spectrum with the killing continuing to the end of the spectrum (2100 A°).

The slides appeared with a rather opaque coating. In the agar was a definite series of transparent lines. These transparent lines, it was believed, indicated the areas in which bacteria had been killed. Whether the transparency was due solely to the absence of live bacteria is questionable because of the remarkable rapidity with which they occurred after the irradiation. Many times this phenomenon was observed in even less than an hour after the removal of the slide from the spectrograph and, at times, it was plainly seen at the end of an hour's exposure. It indicated that some action had taken place between the bacteria, their products, the ultra-violet, and the medium. It is possible that either proteolytic or amylolytic enzymes, or both, are released and act on the medium. Both of these enzymes have been shown to be unharmed by irradiation.

An attempt was made to ascertain whether these lines were due either directly or indirectly to the bacteria. Agar coated slides were covered with bacteria suspended in (a) water, (b) salt-solution, (c) broth. Others were covered with a broth filtered from a twenty-four hour broth culture of organisms. Agar alone was exposed to the radiations in the spectrograph but the spectrum was shown only on the slides containing bacteria.

III

RESULTS

Photographs of the spectra (pages 50 to 54) obtained from Staphylococcus aureus, Staphylococcus albus, Staphylococcus citreus, Bacillus subtilis, and Bacillus dysenteriae (Shiga) are representative of the results obtained from these bacteria. It was not possible to photograph the spectra of Micrococcus catarrhalis, Bacillus paratyphosus B, and Bacillus coli communis, due to the lack of contrast in the slides, although the lines were clearly visible.

The results were as follows:

	Time of Exposure	Longest Killing Wave-Length	Wave-Lengths of Max. Killing
			2800 A°
Staphylococcus aureus . .	1 hr.	3020 A°	2650-2700 A°
			2540 A°
Staphylococcus albus . . .	1 hr.	3020 A°	
Staphylococcus citreus . .	1 hr.	"	
B. Paratyphosus B.	1 hr.	"	Same in All
B. Dysenteriae (Shiga) . .	1 hr.	"	
Micrococcus catarrhalis .	1 hr.	"	
Bacillus colis communis .	1 hr.	"	

---

*The dispersion of the lines as shown in the graphs is not the same throughout. For the sake of clarity a different dispersion was used in the visible and near ultra-violet portions of the spectrum The photographs are all untouched.

Referring again to the table and photographs, it may be seen from the graphs of the intensities of the most pronounced lines in the mercury arc spectrum that, although the lines of wave-lengths longer than 3020 A° are of relatively high intensity, there is no killing beyond this point.

The fact that all wave-lengths shorter than 3020 A°, detectable by our spectrograph and produced by a quartz mercury arc, are bactericidal suggests that the quantum theory is applicable in the case of the bactericidal action of light.

It is interesting to note that the killing of all bacteria studied in these investigations begins at the same wave-length. This indicates that whatever action takes place due to the radiations is the same in all bacteria investigated. The maximum killing wave-lengths are seen to lie at 2800 A°, 2650 A°—2700 A°, and 2540 A° corresponding to the more intense lines in that region of the spectrum.

IV

SUMMARY

1. By means of filters, the killing wave-lengths for the following types of bacteria were roughly determined: Staphylococcus aureus, Staphylococcus albus, Staphylococcus citreus, B. Paratyphus B, Bacillus dysenteriae (Shiga), Micrococcus catarrhalis, Bacillus coli communis.
2. A more accurate determination was made by projecting the spectrum of a quartz mercury arc on bacterial coated agar-covered slides.
3. The longest killing wave-length was found to be the same for all bacteria tested.
4. The quantum theory seems to be applicable in the case of the destruction of bacteria by means of ultra-violet radiations.

This is a preliminary report to be continued with other bacteria and with different types of fungi.

In the graphs (pages 50–54) the numbers represent the wavelengths in A° and the lengths of the lines the corresponding intensities.

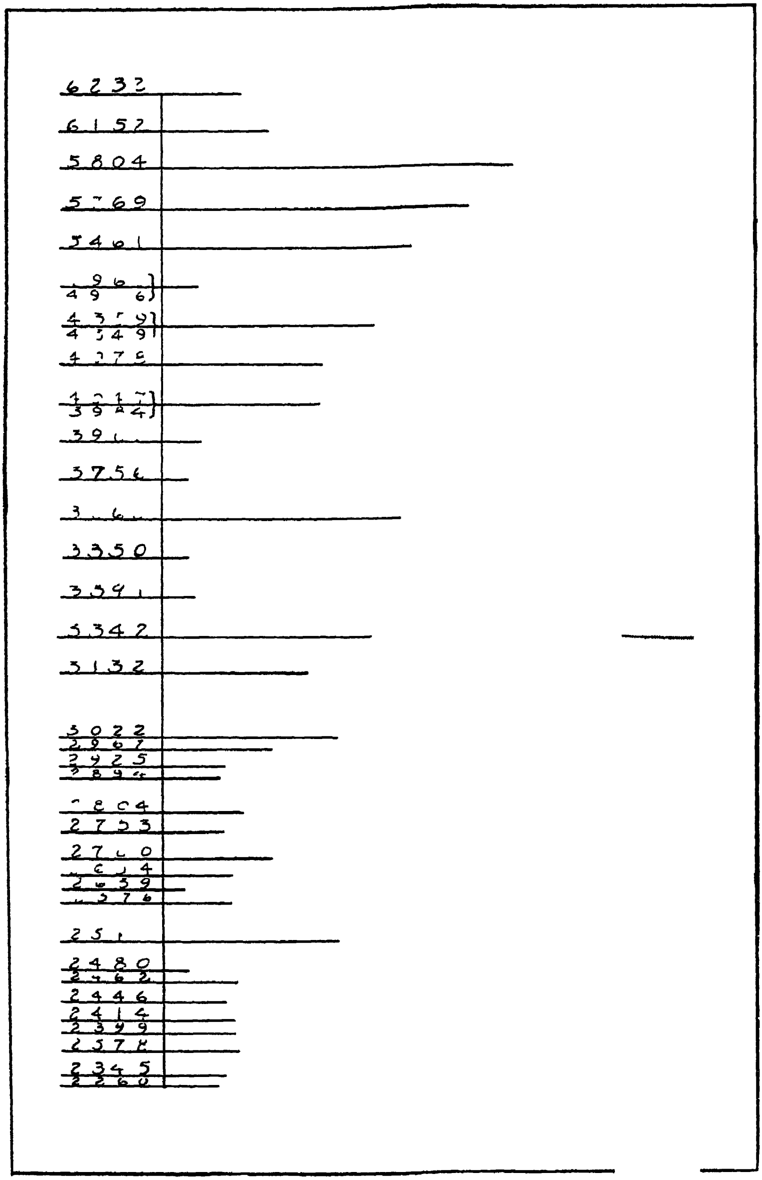

FIGURE I—STAPHYLOCOCCUS AUREUS

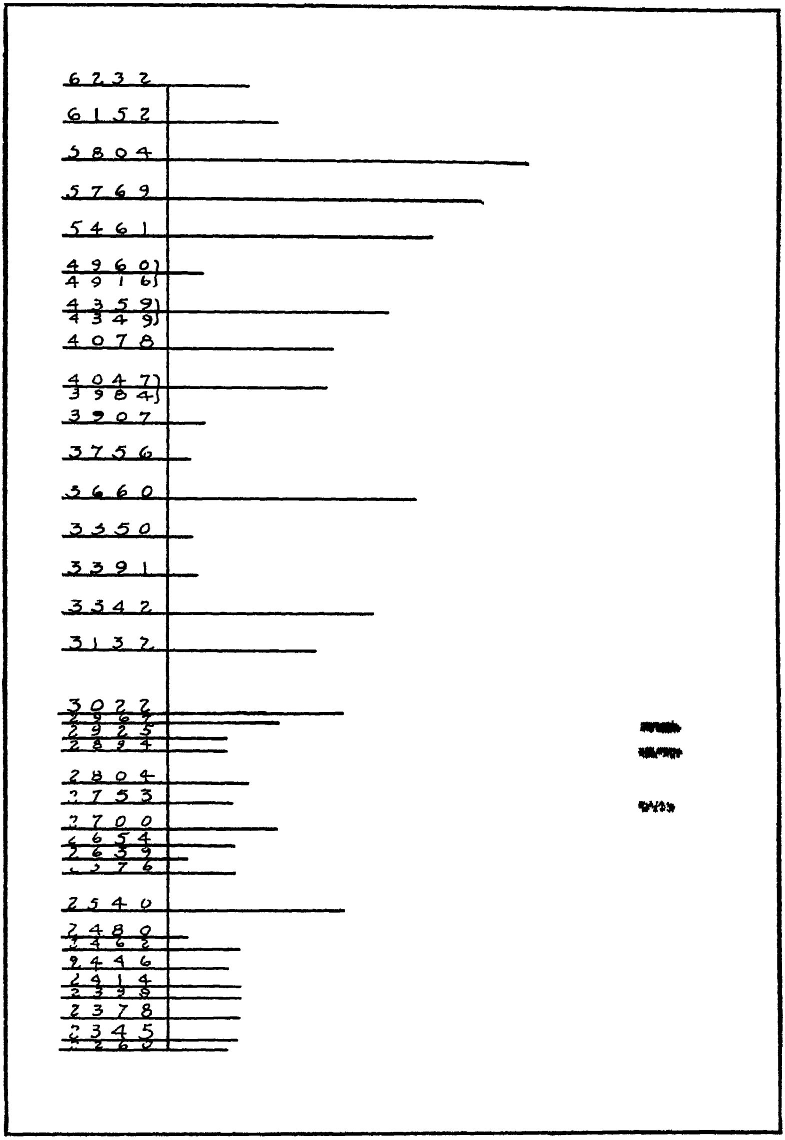

FIGURE II—STAPHYLOCOCCUS CITREUS

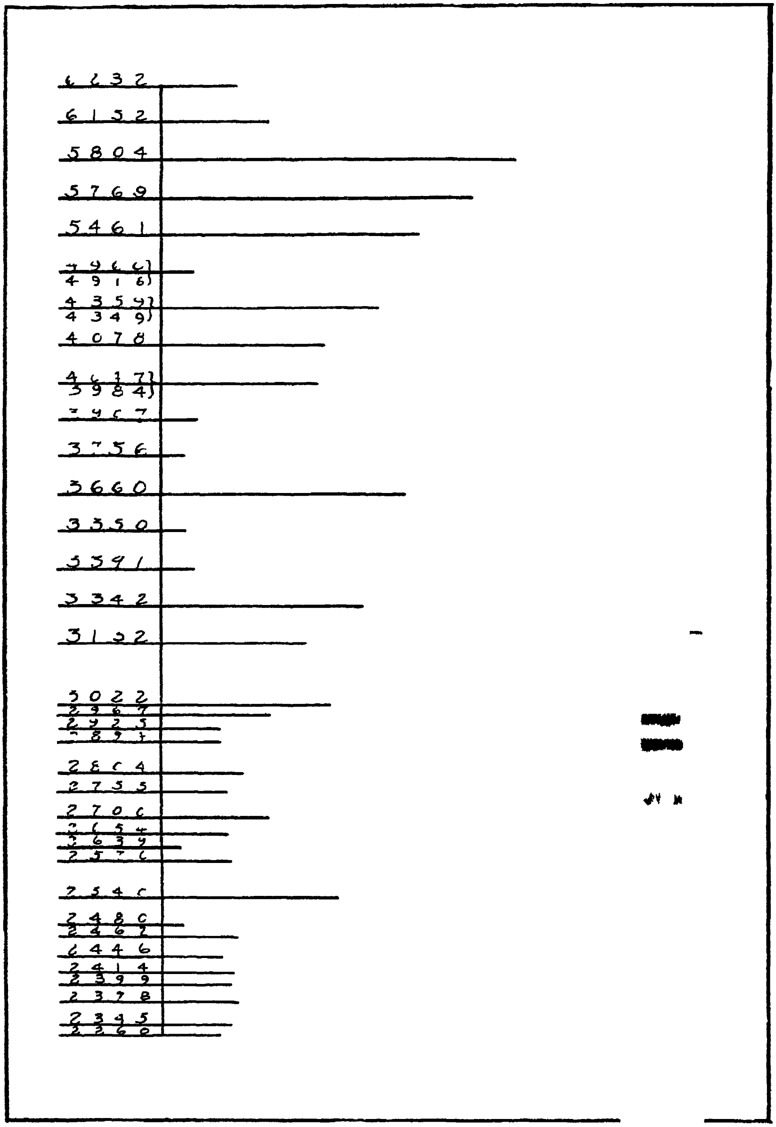

FIGURE III—STAPHYLOCOCCUS ALBUS

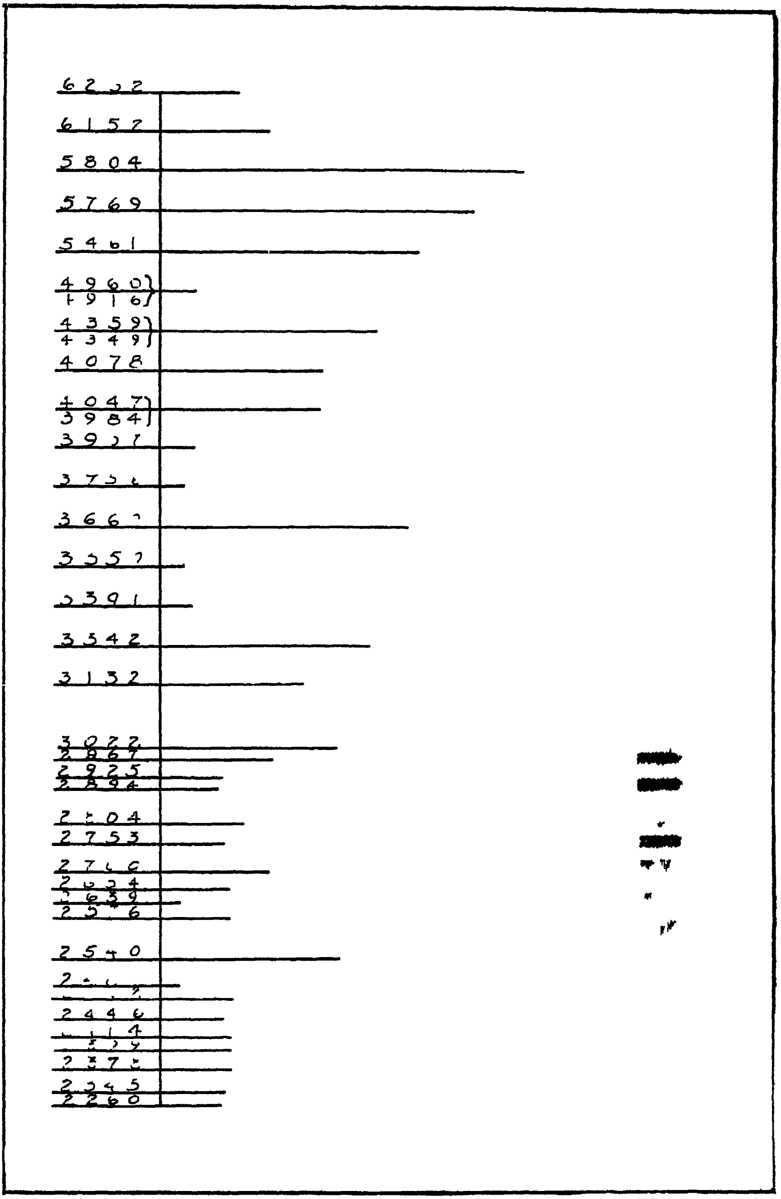

FIGURE IV—B. DYSENTERIAE (SHIGA)

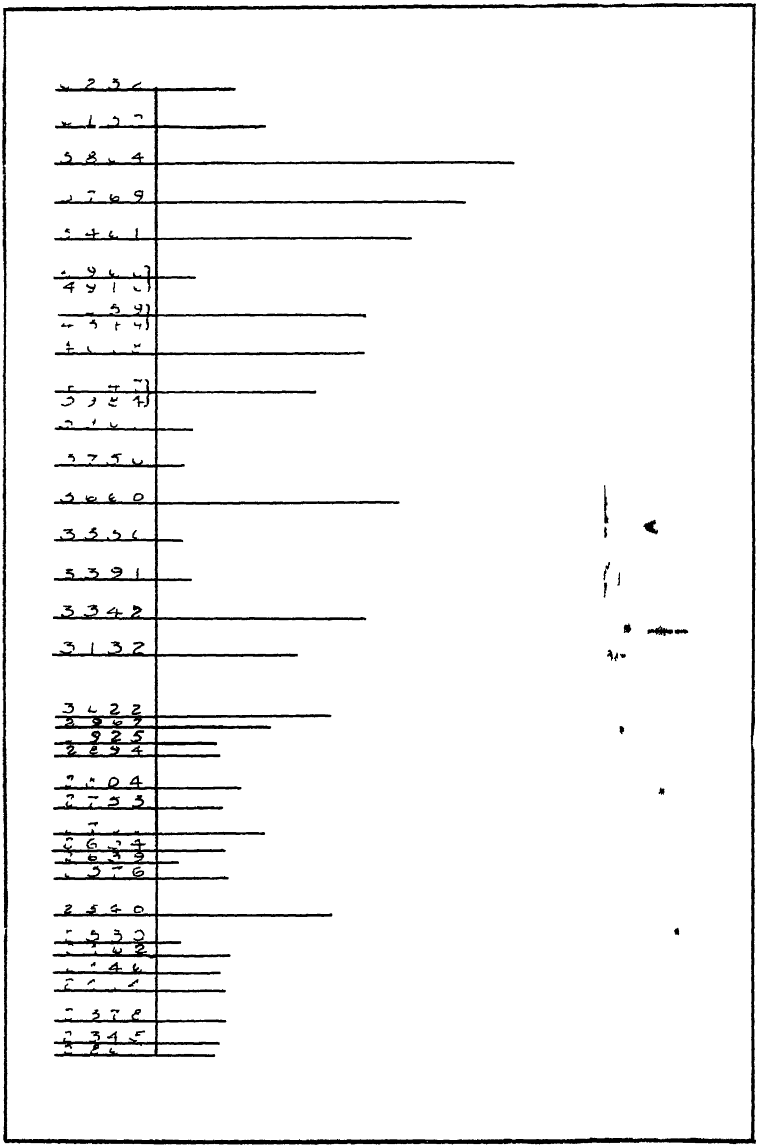

FIGURE V—BACILLUS SUBTILIS

ANTIGENIC VALUE OF VACCINES PRODUCED FROM ULTRA-VIOLET KILLED BACTERIA

By George N. Burger

Determination of the comparative value of vaccines killed by filtered and unfiltered ultra-violet radiations, formalin, and heat was made the object of experiments in this laboratory.

The antigenic value of formalin killed vaccines has been studied by other investigators, notably Wherry and Bowen[1], with results of considerable significance. The action of ultra-violet on bacteria has also been the subject of many experiments. The major part of this work, however, has been done with unfiltered radiation, *i e*, with the full spectrum as emitted by an arc.

From the physicist's viewpoint, the end products of the destruction of bacteria may vary considerably with the type of radiation used. Every wave-length has a definite energy value associated with it as given by the expression

$$E = h\,\upsilon = \frac{h}{\lambda}$$

where E is the energy, h Plank's universal constant, υ the frequency, and λ the wave-length of the radiation in question. From this expression, it is seen that the shorter the wave-length, the greater the energy associated with the wave.

Inasmuch as entirely different actions may be expected from short and long waves (just as with high and low temperatures) it was thought advisable to use the longest bactericidal radiations (minimum energy) for the preparation of vaccines. This, theoretically, should produce a vaccine with the minimum destruction of bacterial products which is possible by means of radiations. The critical wave-length (the longest bactericidal radiation) for the destruction of the bacteria used was determined by exposing the bacteria to radiations from a quartz mercury arc under various filters as described in the paper on "Critical Wave-Lengths in Killing Bacteria" (page 46).

The organism chosen was Bacillus paratyphosus B. This organism is particularly adapted to such experimentation because of its low toxicity towards laboratory animals, and the rapidity with which immune bodies are developed. The immune body used as a comparative agent was the agglutinin.

---

[1] Wherry and Bowen, Jour. Infect Dis XXXVII, 520-522 (1925).

The experiment was planned as follows:

1. To prepare vaccines using four different killing agents.
    - (*a*) Ultra-violet—filtered.
    - (*b*) Ultra-violet—not filtered.
    - (*c*) Formalin.
    - (*d*) Heat.
2. To make all suspensions of the same density.
3. To use rabbits as experimental animals; four for each type of vaccine.
4. To give the same dose to each animal and to give the same amount of increase each time.
5. To test the agglutinating powers of the serums at certain intervals.

I

PREPARATION OF VACCINES

The bacteria were grown on nutrient agar slants for 24 hours and were washed off with physiological salt solution. The washings were filtered through sterile cotton and centrifuged twice to remove any of the dissolved medium. The sediment was then diluted to a certain density standard and placed in test-tubes.

The ultra-violet killed vaccine was prepared in the following manner:

A density standard was made by placing several known dilutions of the bacterial suspension in quartz test-tubes. These tubes were then placed between a mercury arc source and a spectrograph. A fluorescent screen, with the approximate wave-length printed upon it, was placed in the spectrograph. The suspension which allowed the ultra-violet to penetrate to the 2500 A° wave-length was chosen as a standard of density. In this way all of the longer rays, the visible and ultra-violet, down to 2500 A° could penetrate the medium, while the shorter rays were excluded.

Having determined this standard, all of the bacterial suspension was diluted to that degree. This suspension was not as dense as that generally used, but complete killing could not be assured with a more concentrated solution.

Some of the vaccine was then transferred to a series of quartz tubes and these were suspended before a water-cooled, quartz mercury arc lamp for one hour. At intervals the tubes were twirled slightly to insure an uniform turbidity. Following this ½ of a c.c. of the suspension in each tube was planted in nutrient broth which was incubated for three days to determine the complete killing.

The filtered ultra-violet killed vaccines were made up in the same manner except that a quartz cup, containing a water solution of lead acetate and acetic acid, was placed between the lamp and the tube of bacteria. Lead acetate acts as a filter which absorbs ultra-violet rays, the absorption depending on the concentration of the lead acetate. A filter was made which excluded all the rays shorter than 2700 A° which is the critical wave-length for B. Paratyphosus B. All those longer than this were able to penetrate the medium. Infra-red rays were not considered.

The formalinized vaccine was made by treating the bacterial suspension with a 1 to 100 dilution of formalin (40%). The dilution was made by adding 0.1 c.c. of 40% formalin to 9.9. c.c. of the suspension. After standing for twenty-four hours in the incubator, the mixture was washed three times by centrifuging. Its supernatent fluid was then poured off and the residue was made up to 10 c.c. with physiological salt solution.

The heat killed vaccine was held at 63° C for one hour.

Approximately 40 c.c of each type of vaccine were prepared. The test-tubes were capped with rubber test-tube caps and placed in the ice-box. Before using them ½ of a c.c. was drawn from each tube with a sterile syringe and distributed equally into two blood agar slants. One of these was made anaerobic while the other was left under aerobic conditions. These were incubated for five days to make sure that no contaminating organisms were present.

II

AGGLUTINATION TESTS

Sixteen full-grown rabbits were selected. Eight of these were white while the remaining eight were of the Belgian Hare type. Two or 3 c.c. of blood were removed from the ear vein of each rabbit by means of a syringe. The serum was then separated and agglutination tests were prepared using B. Paratyphosus B. as the antigen. Two of the serums agglutinated the bacteria in dilution of 1 to 5, while one of them showed agglutination at 1 to 10.

The primary injection was ¼ of a c.c. given subcutaneously. Three days later ½ of a c.c. was given in the same manner. The third injection was made intravenously, ¼ of a c c. being introduced. All of the subsequent injections were given intravenously. They were increased from ¼ to ½, then ½ to 1 and later to 2 c.c. Each rabbit received 26 c c. of vaccine during the period of injection. This was a great deal more than had been anticipated, and was due to the dilute suspension. More vaccine was prepared as needed.

Because of the excessive crowding, the rabbits fought among themselves Two of them died from injuries; the rest remained well throughout.

Titrations of the serums were made at the end of the experiment, and three months later, to determine the maximum agglutinating titre and the comparative rate of decrease. These are tabulated in Table I (below).

TABLE I.

Date of initial injection—Oct. 27, 1926.
Date of final injection—Feb. 26, 1927.

Rabbit	Type of Vaccine	DATE OF TITRATION	
		March 14 Titre	June 21 Titre
1.	Formalinized	1–5120	1–320
2.	Formalinized	Died	Died
3.	Formalinized	1–1280	1–320
4.	Formalinized	1–2560	1–320
5.	Heat-killed	1–1280	1–160
6.	Heat-killed	1–1280	1–320
7.	Heat-killed.	1–1280	1–160
8.	Heat-killed. . .	1–640	1–320
9.	Ultra-violet, not filtered	1–1280	1–80
10.	Ultra-violet, not filtered	1–1280	1–320
11.	Ultra-violet, not filtered	Died	Died
12.	Ultra-violet, not filtered	1–640	1–160
13.	Ultra-violet, 2700 filtered	1–10, 240	1–320
14.	Ultra-violet, 2700 filtered	1–1280	1–640
15.	Ultra-violet, 2700 filtered	1–2560	1–1280
16.	Ultra-violet, 2700 filtered . .	1–5120	1–640

The titrations, which were set up March 14 (twenty days after the last injections), were constantly higher in the formalinized and filtered ultra-violet killed vaccines than in those killed by heat and unfiltered ultra-violet. Filtered ultra-violet killed vaccines indicated partial agglutination for even greater dilutions than the formalinized vaccines. No definite rule can be drawn as to the decrease in agglutinating power of the serum remaining in the rabbit

The results obtained here have come from only four experimental animals in each case and cannot be taken as proof that one vaccine is better than another in the substances for which tests were made, but they do indicate that bacterial suspensions exposed for one hour to ultra-violet radiations do not lose their antigenic power and that, when a suitable filter is used, the antigenic power may be increased. The experiment is to be repeated with a larger number of animals.

Another point which is, at present, being investigated is the reduction in toxicity of bacterial vaccines by ultra-violet. The work of Dr. Ramon[1] in France and of Drs. Wherry and Bowen in Cincinnati has shown that treating vaccines with formalin reduces the toxicity. Immunity produced in the host as a result of bacterial invasion is always more complete and enduring than that artificially produced. It is possible that killing with ultra-violet is very much different from killing with heat and chemicals so that the antigens are not destroyed while the toxic principles are. A vaccine does not need to be toxic to have a good antigenic power; in fact, the reverse seems to be true. The work of Eberson[2], of St. Louis, on the meningococcus was very encouraging in this respect. An experiment with Bacillus dysenteriae (Shiga) is under way at present in order to determine the above considerations and will be reported later.

III

SUMMARY

1. Bacterial vaccines have been made, using filtered and unfiltered ultra-violet, formalin, and heat as killing agents.

2. The antigenic values have been compared by titrating serum obtained from rabbits injected with these vaccines.

3. The filtered ultra-violet and formalin killed vaccines gave higher titres than did the heat and unfiltered ultra-violet killed vaccines.

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[1] Ramon Paris Med P. 180, Dec. 6 1924
[2] Eberson, J. Immunology, 5 345, July, 1920.

A NEW TYPE OF GALVANOMETER

By J. C. Fardon

The present day galvanometer, though of comparatively high sensitivity, possesses some undesirable features, and as a result it is not fully qualified for routine measurements. In spectroscopic work, where, for instance, from 100 to 500 readings must be taken to cover a desired band of wave-lengths, the time required for these measurements is so extended that it is possible for existing conditions to change and thereby render the readings either inaccurate or entirely beyond utility.

The factors which are greatly detrimental to the readings are the galvanometer zero drift, long period, and susceptibility to external fields. In laboratories where any variety of experiments are conducted, the galvanometer must be protected from such disturbances as vibrations and electrostatic and magnetic fields created by other apparatus Standard galvanometers, in themselves, do not embody the features which would minimize the disturbing effects. The most popular galvanometers, such as the moving coil or moving magnet type, are more readily affected by stray fields and vibrations than the string or Einthoven instrument, but the latter has the disadvantage of being non-linear.

To circumvent these difficulties, a galvanometer more nearly suited to our purposes was designed. The idea originated with Mr. George Sperti and the instrument was designed and built by the author in this laboratory. The new instrument is quite similar in construction to the Einthoven string galvanometer, the essential difference being that the fiber in the galvanometer herein described is free to swing at the bottom while that in the Einthoven galvanometer is rigidly fastened. The advantage of this is that the instrument is linear.

Galvanometer Construction

A general idea of the galvanometer construction may be obtained by observing Figure I (next page).

As shown in Figure I, A indicates the adjustment for moving the silvered quartz fiber in the field of the electro-magnet. B is a window for observing the position of the fiber between the pole faces of the magnet during adjustments. C is a small circular window through

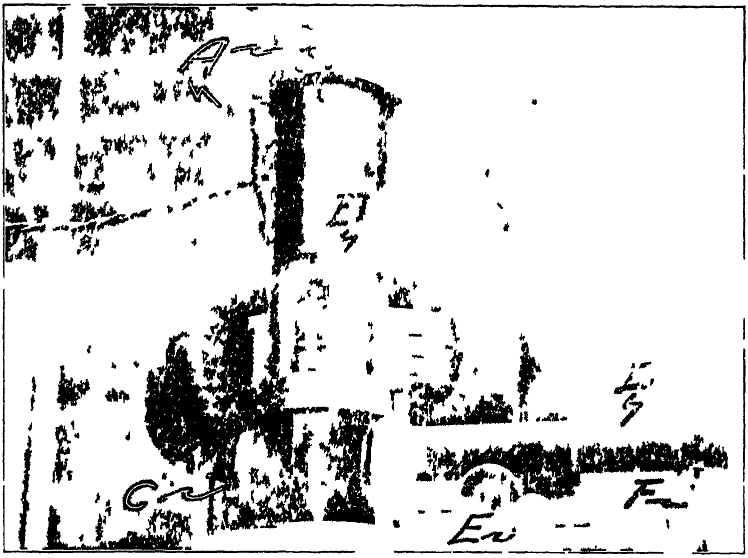

FIGURE I

which light passes from the lamp to illuminate the fiber. E and F are controls fastened to the reading microscope D, and are for adjusting the zero position of the fiber relative to the scale in the microscope ocular and for focussing the fiber. The core of the magnet is composed of 1/32 inch thick laminations. It is 10 inches in diameter, 1 inch wide and 2 inches thick, with an air gap of 2 mm. and a width of pole face of 3 mm. The laminated core supports a total of twelve coils, each with 1384 turns of No. 26 B. & S. gauge copper wire. In the total system three sets of four coils in series are connected in parallel. A current of 1/2 ampere flows through the galvanometer field at a potential of 40 volts. The total number of ampere turns equals 2757. The quartz fiber after being silvered by the Brashears method has a resistance of approximately 150 ohms and a diameter of 20 μ.

Depending upon the magnitude of readings desired, the fiber can be magnified by the microscope to 250, 750 or 1,000 diameters. The silvered fiber is suspended from the adjusting mechanism by a brass rod capable of vertical motion for lowering the fiber into the field. Near the base of the galvanometer and piercing the brass projection tube is an insulated formica bushing through which a terminal is fastened, supporting a small strip of gold foil which completes the circuit to the fiber All apertures in the vertical tube containing the fiber and gold foil are covered with adhesive tape to prevent any external currents of air from disturbing the fiber.

Sensitivity of Instrument

The sensitivity curve of the instrument is shown in Figure II (page 64). The saturation curve is shown in Figure III (page 64), and, as may be observed from the curve, it is possible to obtain a sensitivity corresponding to 10^{-10} amperes per scale division when working on the flat portion of the saturation curve. At present, since no provision is made to cool the coils, advantage is not taken of a higher sensitivity, for the heat generated in the coils causes convection currents to form inside the galvanometer tube, disturbing the motion of the fiber.

Summary of Data

Current sensitivity—10^{-9} amperes per scale division.

Total number of NI—2757.

Voltage applied to field—40 volts.

Size of silvered fiber—20 μ.

Period of full scale deflection—1 second.

Resistance of galvanometer field—80 ohms.

Resistance of fiber—150 ohms.

Magnification—250 diameters

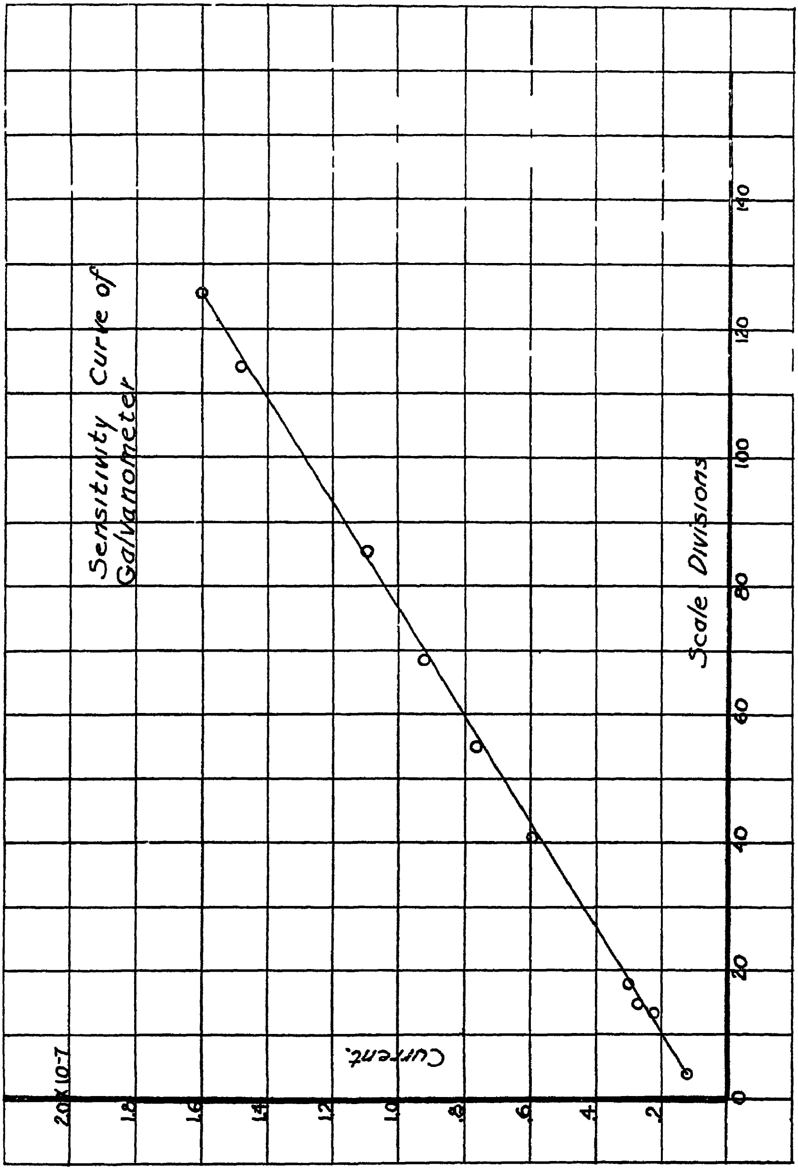
Sensitivity Curve of Galvanometer
Scale Divisions
0
20
40
60
80
100
120
140
Current.
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0 X 10-7

FIGURE II

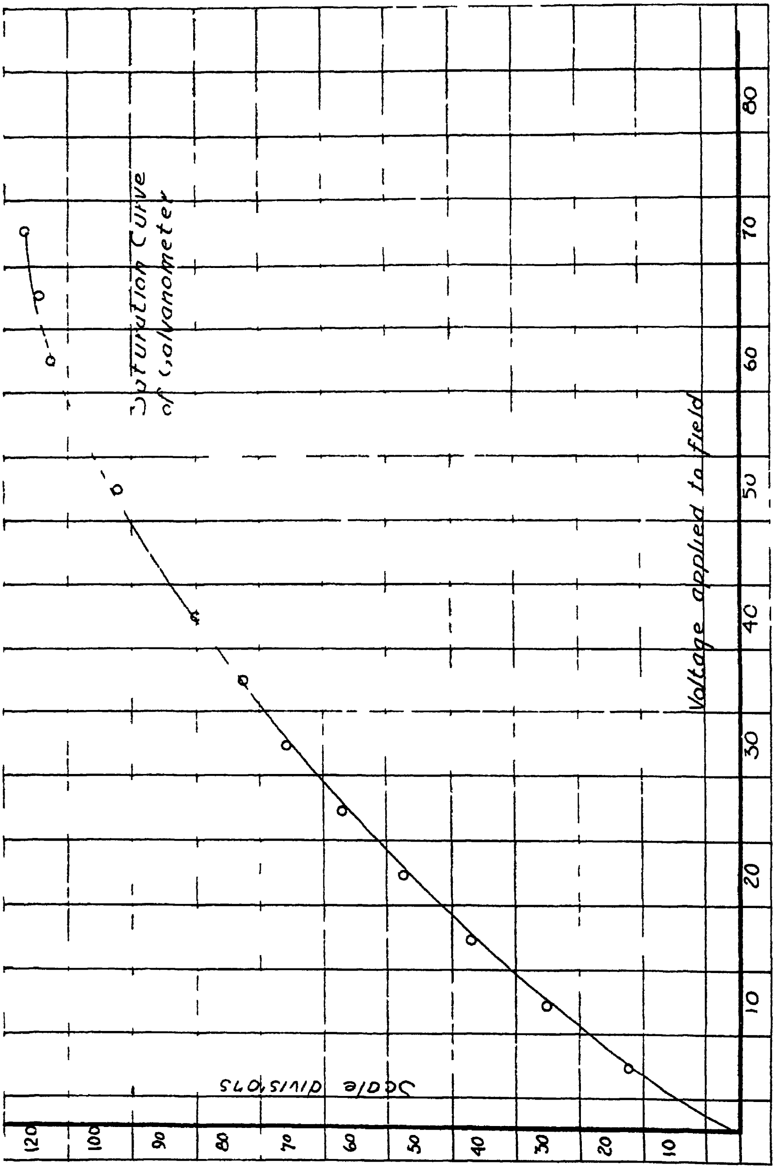
Saturation Curve
of Galvanometer
Voltage applied to field
Scale divisions
10
20
30
40
50
60
70
80
10
20
30
40
50
60
70
80
90
100
120

FIGURE III

ADAPTATION OF THE INFRA-RED SPECTROMETER AND ITS ACCESSORIES TO BIOLOGICAL PURPOSES

By J. C. Fardon

Spectroscopy has long been known as one of the most valuable tools of the physicist as well as of the chemist. An adaptation of the infra-red spectrometer and its accessories for the biologist is the subject of this paper.

In the X-ray region, the absorption and emission spectra are characteristic of the atoms, while in the ultra-violet, visible, and infra-red, the absorption spectra are especially characteristic of molecular structure. Coblentz[1] has shown that definite groupings of molecules have characteristic absorption peaks in the infra-red region of the spectrum. J. W. Ellis[2] has shown the ease with which the CH series could be identified in compounds containing this series. R. S. Mullikin[3] and E. K. Plyler[4] and others have shown the possibilities of determining the isotopes of some of the elements through infra-red spectroscopy.

Very little work has been done on the determination of characteristic absorption spectra of biological materials. Many difficulties were encountered while working with such materials, inasmuch as their characteristics varied with time. This necessitates taking readings with extreme rapidity.

With these points in view, a spectroscopic unit as described herein was designed and built.

In Figure I (page 66) is given a diagrammatic representation of the spectrometer and its accessories. In order to eliminate convection currents and outside radiations, the spectrometer, thermopile, and infra-red source were placed in a casting which could be evacuated to a pressure of 150 microns. The casting was connected to the pump by means of a tube of large diameter as shown at (*a*).

In this apparatus the air-tight absorption cell (*c*) is controlled from the outside of the casting by a system of pulleys (*n*) and (*h*). The source of the infra-red is held inside of a water-cooled chamber which is connected to the casting by a tube (*e*).

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[1] Coblentz, W W, Investigation of Infra red Spectra (1905)
[2] Ellis J W, Physical Review July 1926
[3] Mulliken, R S, Physical Review, March 1925
[4] Plyler, E K, Physical Review, August, 1926

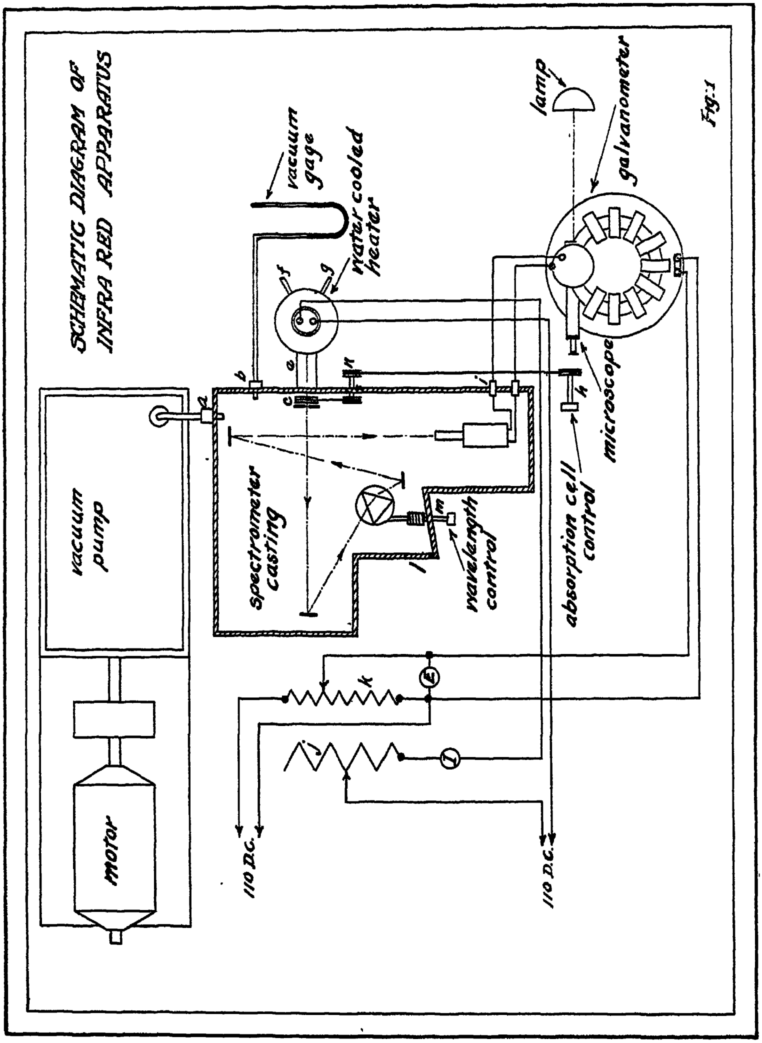
SCHEMATIC DIAGRAM OF
INFRA RED APPARATUS
vacuum pump
motor
spectrometer casting
vacuum gage
water cooled heater
lamp
galvanometer
microscope
absorption cell control
wavelength control
Fig: 1
110 D.C.
110 D.C.
a
b
c
e
f
g
h
i
j
k
l
m
n
E
I

FIGURE I

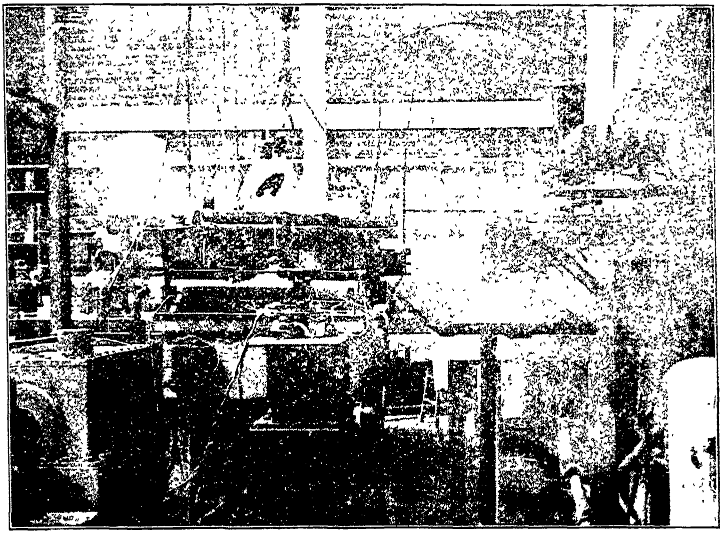

FIGURE II

The wave-length drum on the spectrometer which is controlled by the knob (*m*) is observed through a window (*l*) mounted and connected to the casting. The thermopile leads are brought to two terminals (*i*) mounted in the wall of the casting and from there are

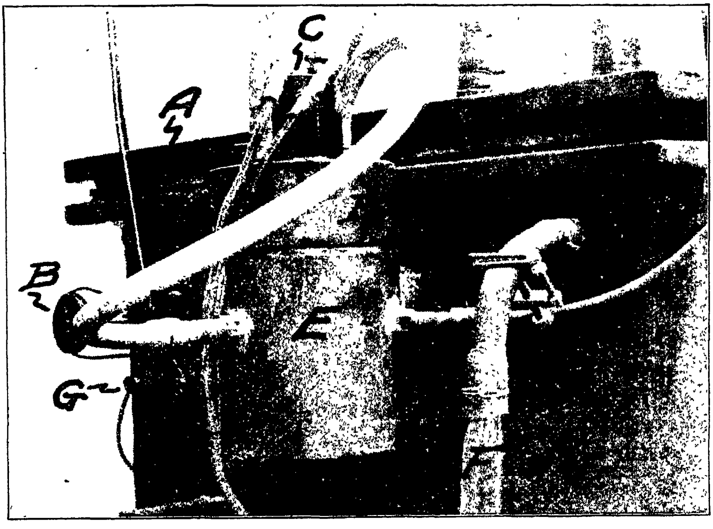

FIGURE III

connected directly to the galvanometer with brass wire. Since the terminals on both casting and galvanometer are of brass it was found best to use a conductor of the same material in order to minimize currents due to thermo-junctions of dissimilar metals.

The galvanometer was mounted upon a bracket which was rigidly fastened to the wall as shown in Figure II (page 67). Although the laboratory is situated on the third floor of the building, the nature of the galvanometer is such that the vibrations of the wall upon which the instrument was mounted did not seriously affect the accuracy of the readings.

I

DESCRIPTION OF APPARATUS

1. *Vacuum Pump*: The Adam Hilger Trimount vacuum pump is used for evacuating the spectrometer casting The pump, when connected directly to a vacuum gauge, indicates a pressure of 5 μ. When the pump is connected to the casting, a pressure of 150 to 200 μ can be obtained in approximately five minutes, the capacity of the casting being about 32 liters.

2. *Vacuum Gauge*: The later model Cenco vacuum gauge is used for indication of pressure in the casting. The range of the gauge in microns of pressure is 450 to 0.5 μ.

3. *Spectrometer*: The spectrometer is of the Adam Hilger make with a rock salt prism and self-contained thermopile, the wave-length drum being calibrated for a range of wave-length from 0.5 μ to 10 μ.

4. *Spectrometer Casting*: The casting containing the spectrometer is of cast brass of 1/2 inch walls, provided with a cover of the same material. The casting and cover are made with flanges which are machined to accommodate a 1/4 inch thick rubber gasket to insure an air-tight fit. There are two ground-in, tapered holes in the casting which receive the tapered pins that connect to the spectrometer inside and are controlled outside the casting with the aid of knobs. Directly opposite the wave-length drum of the spectrometer is located a 1/4 inch glass window held in place against a ground aperture in the casting by DeKotinsky cement. As shown in Figure I, (*a*) and (*b*) are two tubular brass inlets threaded and cemented into the casting, one leading to the pump, the other connected to the vacuum gauge. On the cover of the casting is an air inlet provided with a stop-cock and a bottle of calcium chloride for absorbing any moisture which may be present in the air, thereby preventing any water vapor from condensing on the rock salt prism. Figure II, A, indicates the calcium chloride container mounted on the casting cover.

In Figure I (*i*) and Figure III (page 67) G, may be seen the terminals which are insulated from the casting by hard rubber bushings and which serve to connect the thermopile to the galvanometer fiber. The entire casting is covered with a number of coats of shellac as a precaution against possible leaks. All air connections leading to and from the casting are of special air-tight rubber tubing, wired and of the shortest possible length.

5. *Chamber Containing Infra-Red Source*: The container for the heating unit is machined of brass and is connected to the casting by a rigid brass tube which also serves the purpose of passing the radiations from the source to the collimator slit of the spectrometer. By unscrewing six screws, the cover, Figure III, D, may be taken off of the container, E, and the heating unit replaced. A circular rubber gasket is here also used to insure an air-tight fit between cover and container. Both cover and container are double walled and joined by a piece of hose for water-cooling, thereby preventing the melting of cemented and soldered joints. As may be seen in Figure III, C is an oil-cooled cup through which pass two terminals connecting the heating unit. The terminals are insulated from the cover by hard rubber bushings and DeKotinsky cement. The oil in the cup also serves as an air seal around the terminals. The heating unit proper is fastened on the terminals with nichrome wire and is made of a compound containing carbon and graphite in the form of a rod ¼ inch in diameter and approximately two inches long.

6. *Galvanometer* The new type galvanometer is described in full in the preceding article, (page 60).

7. *Absorption Cell*: The cell, the position of which is indicated in Figure I (*c*) is constructed of brass. The frame which supports the quartz windows has an aperture measuring 6 by 12 mm. There are two flanges lengthwise on either side of the brass frame. These flanges slide in a carrier on the spectrometer to guide the travel of the cell in front of the collimator slit. The quartz windows for the cell measure about 8 by 14 mm. and are 1 mm. thick. The two plates of quartz are separated by a small frame or washer of lead foil 0.1 mm. thick and cemented together by DeKotinsky cement. Two grooves are filed in each plate, on top and bottom, parallel to the surface, in such a manner that when the two plates are pressed together with the lead washer between them, two small holes appear on either side of the cell into which the two capillary glass tubes are cemented, thus making it possible to fill and clean the cell. The two quartz plates which are cemented together are in turn cemented to the brass car-

rying frame. The cell is filled and cleaned with the aid of an hypodermic syringe, the needle of which is inserted into the capillary tubes The thickness of the liquid in the cell equals 0.1 mm. and exposes a surface of 72 sq. mm. to the radiations from the source.

II

INFRA-RED SOURCES

Figure IV (page 71) shows the emission from a "compound" conductive rod containing carbon and graphite, and of a coil of nichrome wire of No. 22 B. & S. gauge. As may be seen from the curves, the rod at two amperes has a greater intensity than the nichrome source at seven amperes. The nichrome wire was operated at a temperature very near its melting point. The wire operating under this condition melted frequently, making the use of this material quite undesirable as a source of infra-red radiations. The graphite rod, on the other hand, failed to burn out even at temperatures considerably higher than were necessary to operate.

Both emission curves indicate a peak at 2.5 μ and a fair intensity out to 4.5 μ If the temperature of the sources be decreased, the peak shifts according to Wien's displacement law, given by the equation

$$K = \lambda T$$

where K is a constant, λ the wave-length of the peak due to black body radiation, and T the temperature of the source. From this equation, it can be seen that tungsten which has a melting point of 2979° C is ideal as a source in the visible and near infra-red portions of the spectrum.

By evacuating the casting, tungsten was studied in this portion of the spectrum and found to be very satisfactory.

Among other sources investigated may be mentioned nichrome wire wound on quartz tubing, nichrome wire coated with porcelain cement, Nernst Glowers, and Nernst Heaters, none of which proved as satisfactory as the tungsten wire or graphite rod. By the use of a concave mirror placed behind the source, thereby focussing the radiations on the slit, the galvanometer readings were increased by an order. The disadvantage of this method, however, was the deposit of films on the surface of the mirror causing inconsistencies during readings. It is believed that these difficulties will be overcome in the near future. All possible steps are being taken to procure the most intense source of radiations throughout the entire spectrum so as to permit the use of a relatively low sensitivity of the galvanometer.

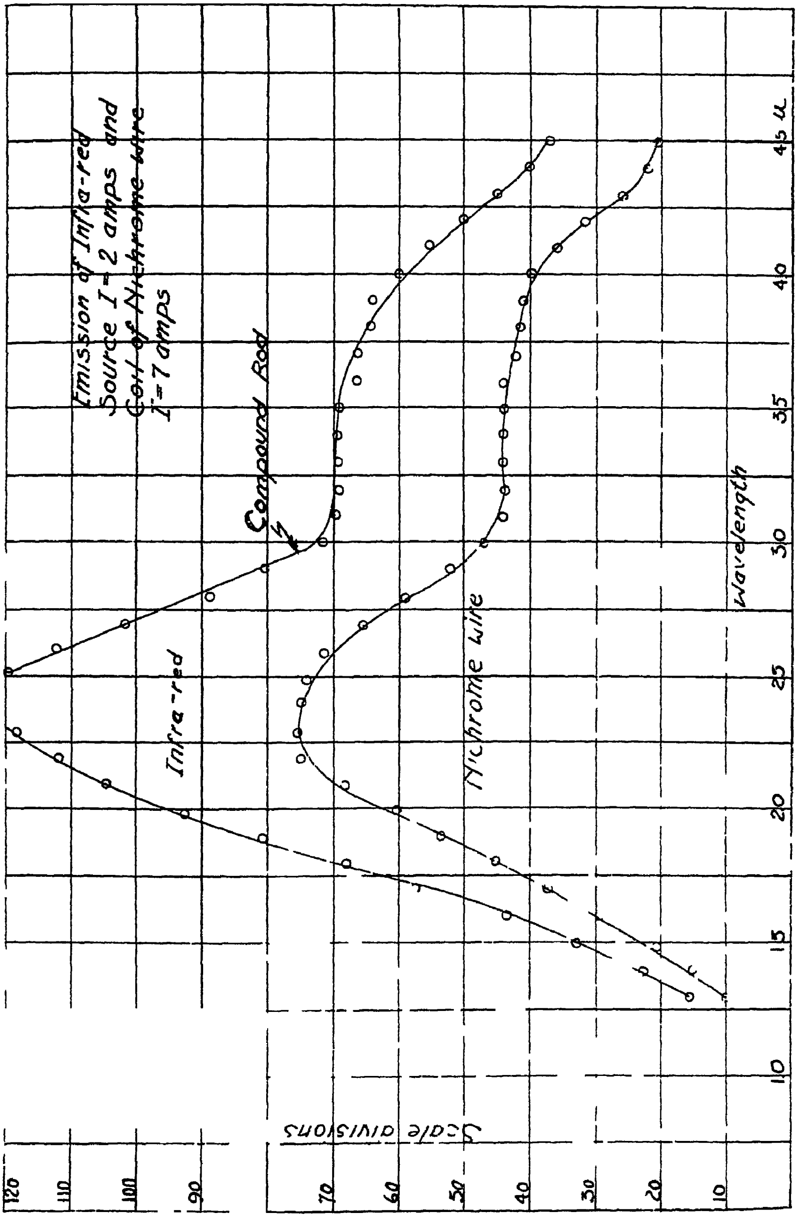
Emission of Infra-red Source I = 2 amps and Coil of Nichrome Wire I = 7 amps
Compound Rod
Infra-red
Nichrome Wire
Wavelength
10
15
20
25
30
35
40
45 µ
Scale divisions
10
20
30
40
50
60
70
90
100
110
120

FIGURE IV

INFRA-RED ABSORPTION SPECTRA OF BIOLOGICAL MATERIALS

By J. C. Fardon

The infra-red absorption spectra of various organic and inorganic compounds has already been obtained and studied by numerous investigators, with the result that it now enables one to predict certain absorption peaks due to the grouping of atoms in the compound.

Coblentz[1], for instance, has shown the possibilities of the use of infra-red spectroscopy in determining the cases in which water exists as water of crystallization in the sugars. By merely identifying peaks in the absorption spectra, it is possible to secure information which heretofore has not been within the scope of the chemist.

Little or no work has been done yet, however, towards employing this type of spectra in determining structural differences in various groupings of the amino-acids in proteins. Previous investigations and theorizing indicate that experiments along this line may well be expected to lead to successful micro-analysis of some biological materials, the structure of which is unknown today. It is with a desire to make this contribution to micro-biological analysis that the laboratory has begun the series of preliminary investigations described here.

When experimenting was begun, fluorite plates were not available for absorption cells and as a consequence only a rough survey of the near infra-red region was made. No attempt was made to operate the galvanometer at its highest sensitivity, to evacuate the spectrometer case, or to close the slits to their minimum value, inasmuch as the work is to be repeated with a fluorite cell.

The present work was carried on merely to determine whether sufficient transmission of the biological materials exists to permit the use of infra-red spectroscopy for qualitative analysis. It is, for instance, well known that H_2O absorbs so strongly beyond 2 μ that direct absorption spectra of the proteins is extremely difficult, if at all possible.

Throughout the readings taken in the present investigation, the spectrometer slits were kept at 0.5 mm. The source of radiation used was a conductive rod containing carbon and graphite with a current of from two to three amperes flowing through it. The absorption cell was of quartz and when filled, supported a layer of liquid

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[1] Coblentz, Investigation of Infra-red Spectra (1905).

0.1 mm. thick. The range of wave-lengths embraced in the present investigations of absorption spectra was from 1.3 μ to 4.5 μ.

The transmission curve of the quartz cell used is shown in Figure I (page 74). As may be readily seen, the transmission of quartz was very favorable from the region of 1.3 μ to about 4 μ. Quartz became entirely opaque at 5 μ. The slight depression near 3 μ was checked against curves of other investigators and found to be characteristic of quartz. At the time the readings were taken, polished pieces of fluorite were not at hand.

Transmission curves of cod-liver, aniseed, lemon, and cinnamon oils are shown in Figures II, III, IV, and V (pages 75 to 78). Each shows a decided absorption peak at 3.5 μ, which, by a study of their structure and by comparison to other curves of organic compounds, indicates that they are responsible to the CH_3 group.

Figure VI (page 79) gives the transmission of cinnamon oil which has recently been obtained and is here shown to increase in transmission after reaching the 3.5 μ peak, whereas the old sample of the oil which had been standing around the laboratory was completely opaque beyond 3.5 μ. The behavior of the latter oil may be due to the accumulation of impurities or to decomposition.

From Figure VII (page 80) may be seen the characteristic transmission of a layer of gelatin 0.05 mm. thick. The peak due to the CH_3 groups shifted slightly towards the lower end of the spectrum, and also slight depressions were manifested near 1.5 μ and 2 μ From a comparison of the transmission of gelatin with that of H_2O (as obtained by Coblentz) it seems that these peaks occurring at 1.5 μ, 2 μ and 3μ were due to the H_2O present in gelatin.

Some of the formulae which are included to indicate the CH_3 groups, although they are not all definitely established, appear on page 81.

SUMMARY

1. A difference in the absorption spectra of the two samples of cinnamon oil clearly indicates a difference in their structure or the presence of foreign substances in one.

2. Absorption curves have been obtained for the region from 1.3 μ to 4.5 μ for gelatin and cod-liver, aniseed, lemon, and cinnamon oils.

It is anticipated that after the completion of the present range of 0.5 μ to 10 μ and the range of 10 μ to 100 μ (the spectrometer for which is now being designed) some inter-

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*For full description of galvanometer and layout used throughout this work, see pages 60 and 65.

esting data will result. Perhaps contributions of merit will be obtained in the use of infra-red spectroscopy for the determination of complex proteins and for the differentiation of various chemical products of diseases such as serum from cancerous and non-cancerous subjects, etc.

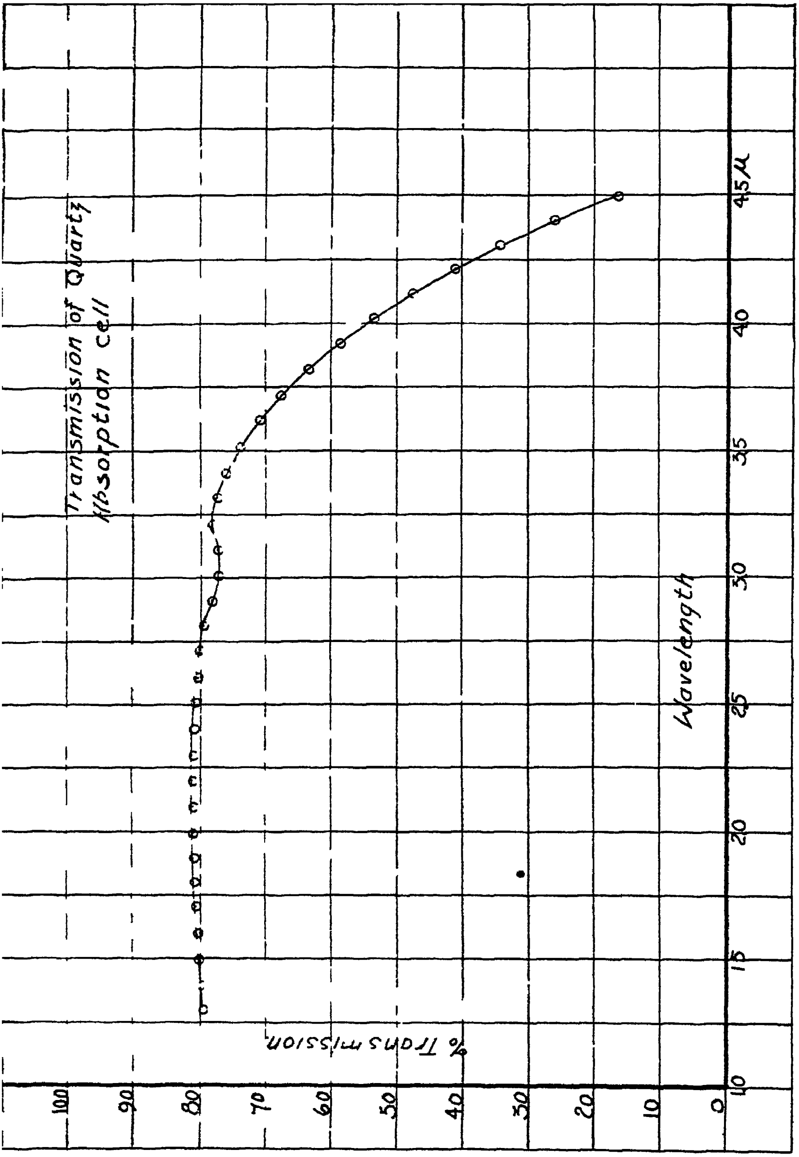

FIGURE I

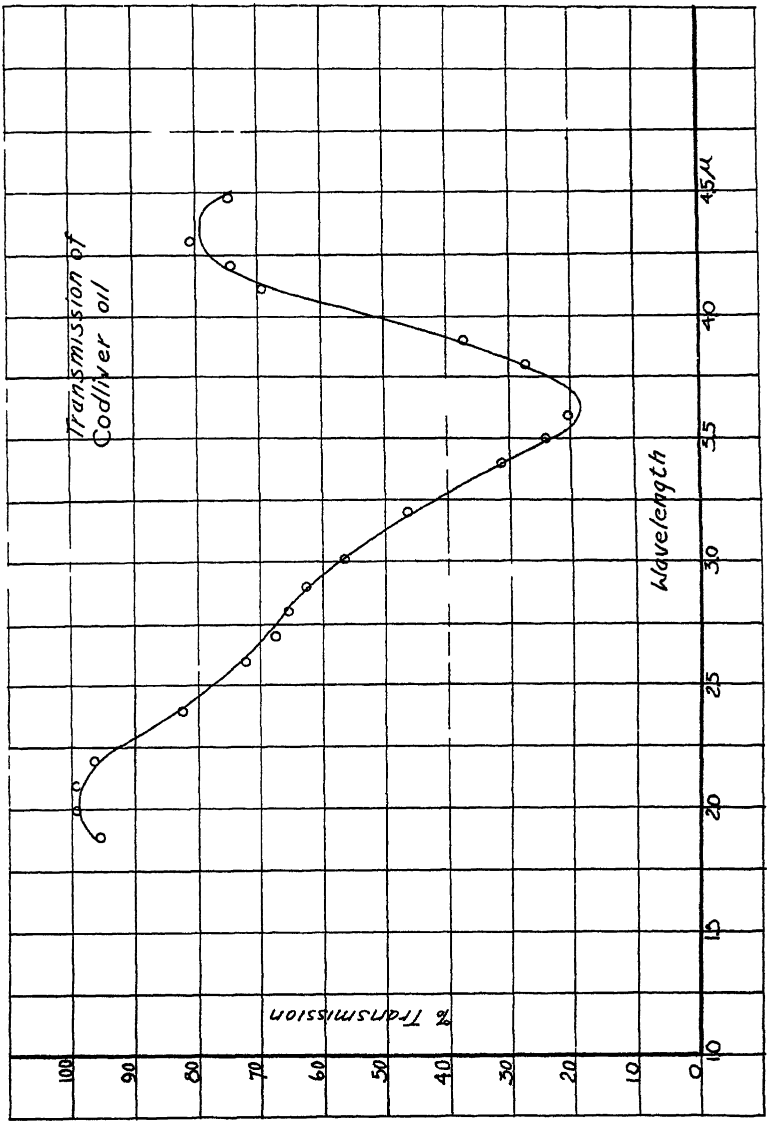
Transmission of
Codliver oil
Wavelength
% Transmission
100
90
80
70
60
50
40
30
20
10
0
10
15
20
25
30
35
40
45μ

FIGURE II

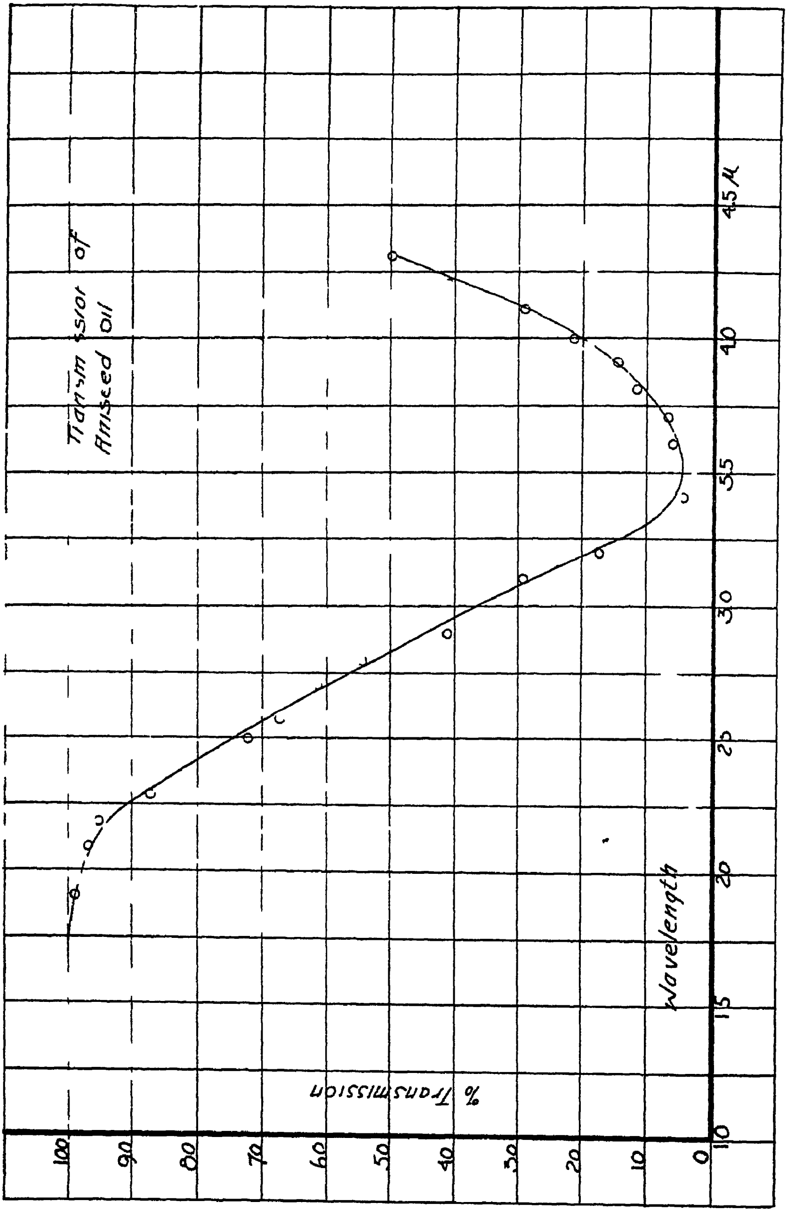
Transmission of Aniseed Oil
% Transmission
Wavelength
100
90
80
70
60
50
40
30
20
10
0
10
15
20
25
30
3.5
40
4.5 μ

FIGURE III

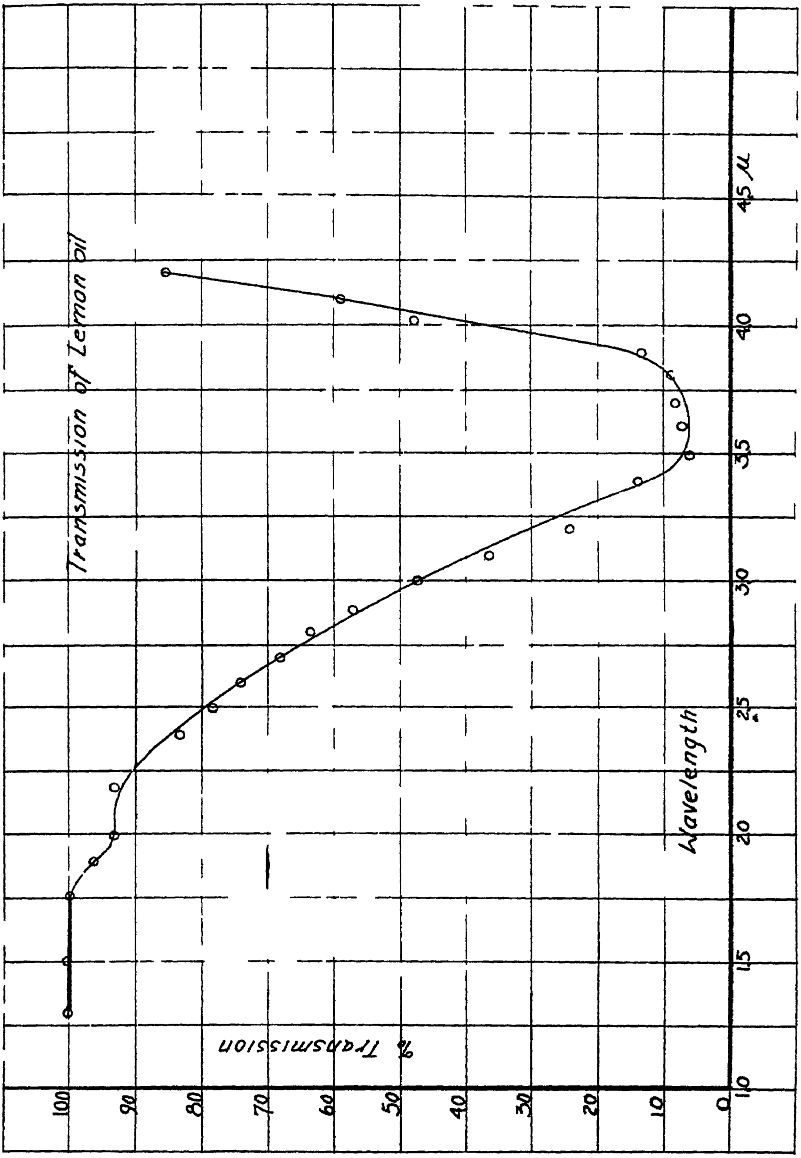
Transmission of Lemon oil
% Transmission
Wavelength
100
90
80
70
60
50
40
30
20
10
0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5 μ

FIGURE IV

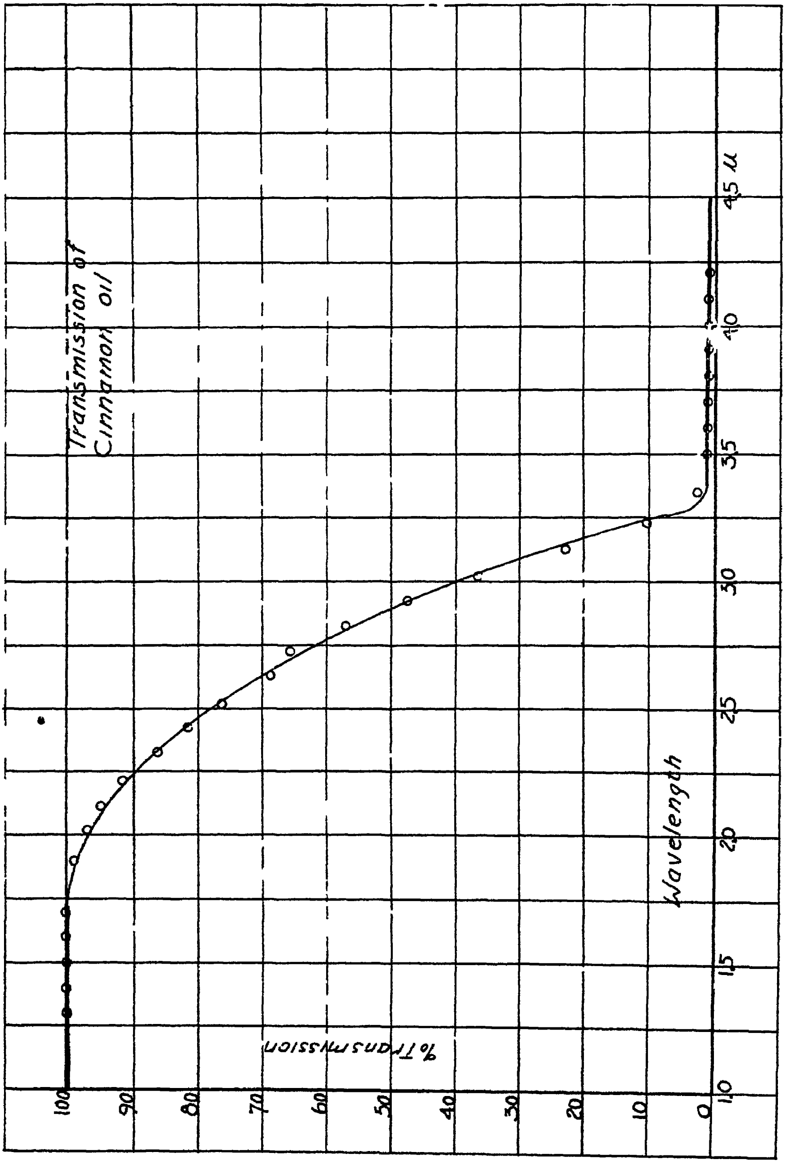
Transmission of Cinnamon oil
% Transmission
100
90
80
70
60
50
40
30
20
10
0
Wavelength
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5 μ

FIGURE V

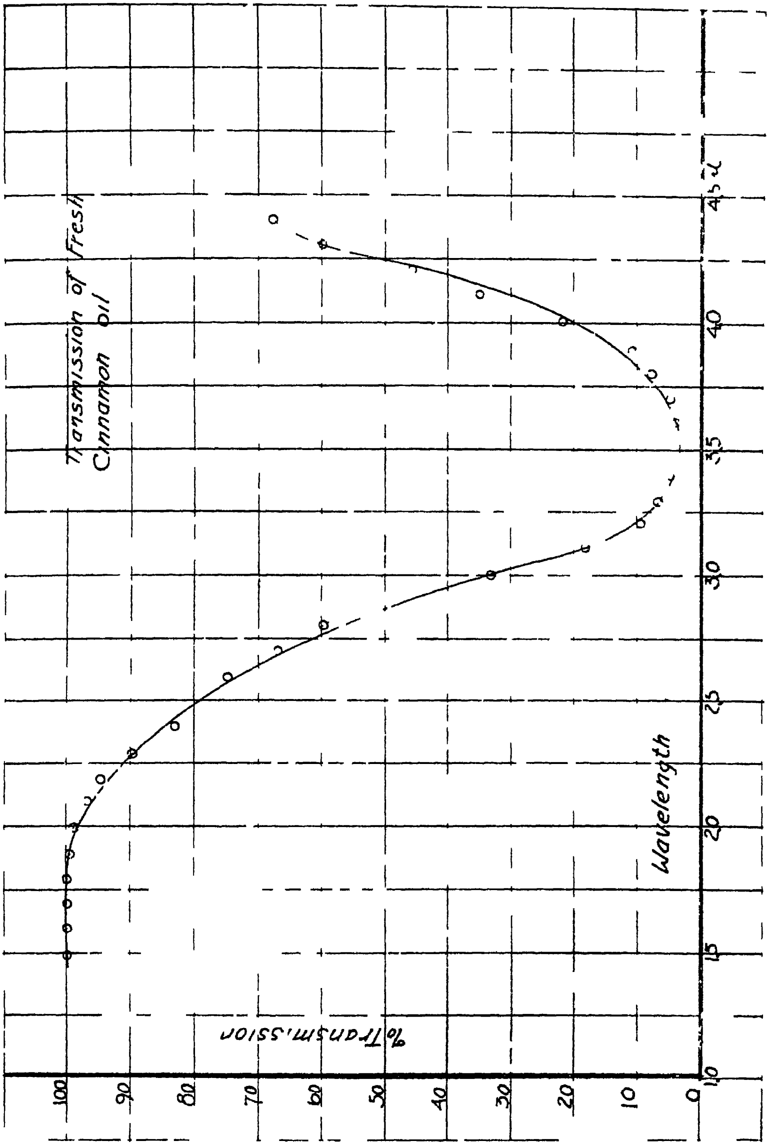
Transmission of Fresh Cinnamon oil
Wavelength
% Transmission
100
90
80
70
60
50
40
30
20
10
0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5 μ

FIGURE VI

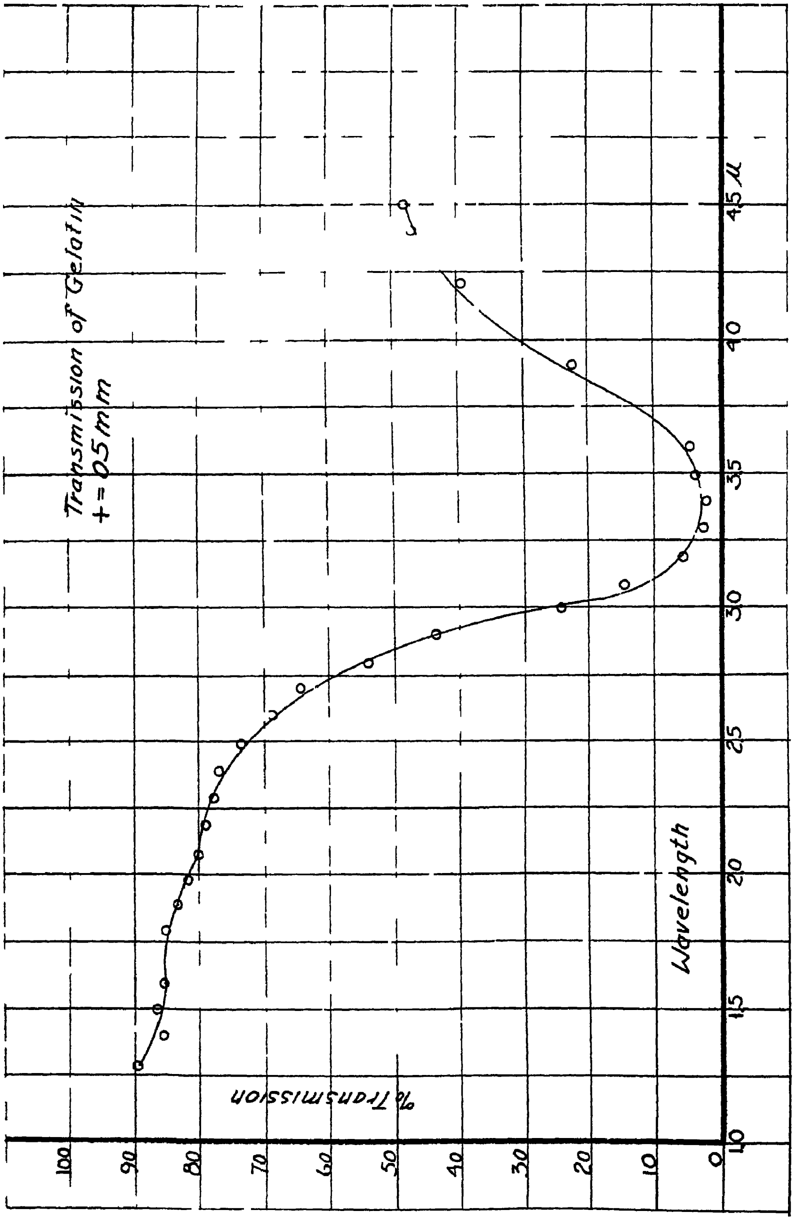
Transmission of Gelatin
t = .05 mm
Wavelength
% Transmission
100
90
80
70
60
50
40
30
20
10
0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5 μ

FIGURE VII

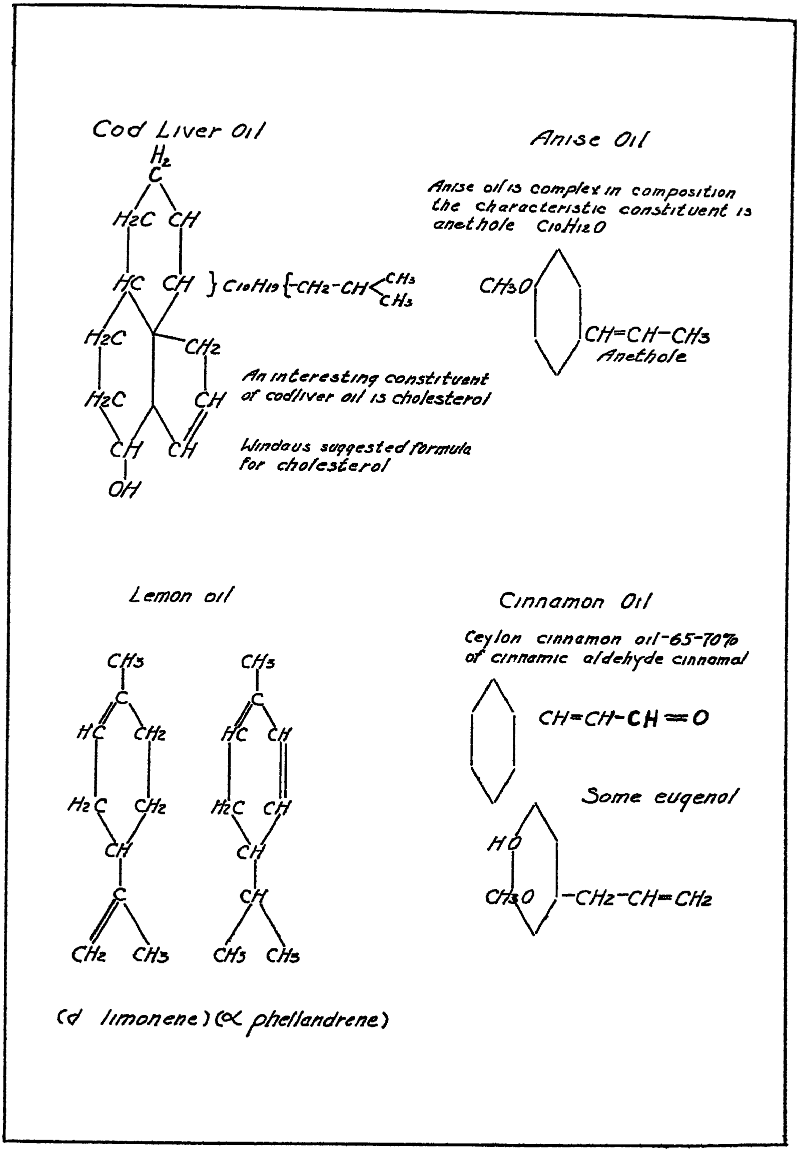
Cod Liver Oil
H2
C
H2C CH
HC CH } C10H19 {-CH2-CH<CH3 CH3
H2C CH2
H2C CH
CH CH
OH
An interesting constituent of codliver oil is cholesterol
Windaus suggested formula for cholesterol
Anise Oil
Anise oil is complex in composition the characteristic constituent is anethole C10H12O
CH3O
CH=CH-CH3
Anethole
Lemon oil
CH3 CH3
C C
HC CH2 HC CH
H2C CH2 H2C CH
CH CH
C CH
CH2 CH3 CH3 CH3
(d limonene) (α phellandrene)
Cinnamon Oil
Ceylon cinnamon oil-65-70% of cinnamic aldehyde cinnamal
CH=CH-CH=O
Some eugenol
HO
CH3O
-CH2-CH=CH2

FIGURE VIII

SOME POSTULATES ON ATOMIC AND MOLECULAR STRUCTURE AND ON ATOMIC AND MOLECULAR ABSORPTIONS AND EMISSIONS OF ENERGY

By HERMAN SCHNEIDER, Sc.D.

(*Reprinted from the April,* 1927, *Issue of the Co-operative Engineer.*)

The following are assumed:

a. The Bohr atom of hydrogen.

b. All atoms are in motion.

c. All atoms and molecules move with changes of velocity.

d. All atoms are magnetic.

e. A fluctuating electrostatic field emits electromagnetic waves.

Accepting these, the following postulates are presented:

1. If an atom of hydrogen moves (with changes of velocity) in a direction lying in the plane of the orbit, its orbit will become a varying ellipse. The electrostatic stresses within the atom will vary. Hence the atom will emit electromagnetic waves (or energy) just as a radio oscillator emits waves. This energy must be supplied. Therefore, translation in the plane of the orbit, with varying speeds of the proton, requires that some of this energy be used for this emission.

2. If the atom moves in a direction perpendicular to the plane of the orbit, the orbit remains circular, the path of the electron being helical. Hence, there will be no variation (or at the most, relatively slight variations) of the electrostatic stresses between proton and electron, and hence no (or relatively small) emission of energy. Less energy would be needed, therefore, to move the atom in a direction perpendicular to the plane of the orbit than to move it in a direction lying in the plane of the orbit.

3. The principle of least energy would then require all free movement to be perpendicular to the orbital plane.

4. All of the orbital electrons in any free atom must lie in a plane, for if they did not, motion in the direction of least resistance would

require additional energy for propulsion over that which would be required if they were all in one plane.

5. For motion in any direction other than perpendicular to the plane of the orbit, the K, L, M,—orbits would initially be circular. For if any were initially elliptical, a still greater amount of energy would be radiated and hence required.

6. Similarly, for motion perpendicular to the orbital plane, the principle of least energy would require all of the orbits of a free atom initially to be circular. For if any were elliptical, there would again be greater variations in the electrostatic stresses within the atom.

7. If an atom moves in a magnetic field which inclines the orbital plane from a direction perpendicular to the direction of motion, the orbits will become elliptical and energy will be emitted. The degree of ellipticity will depend upon the inclination.

8. If the inclination of the orbital plane to the direction of motion is sufficiently small, the resultant force upon an electron could conceivably eject it from its orbit. This would explain the "quantum" ejection of electrons, the stripping of atoms in hot stars, etc. It assumes (and leaves begging) the postulate of fixed (K, L, M,—) orbits.

9. The ejection of an electron from its orbit under these circumstances, would result in a sudden release or emission of energy; since this energy would no longer be required. The effect would be similar to that of a spring in tension suddenly released. The tension would be a "field" tension.

10. Conversely, the absorption of energy (*e. g.* electromagnetic) would be the use of that energy to drive electrons whose orbital plane is inclined, by the impinging (electromagnetic) energy, to the direction of motion. The energy so absorbed is emitted, (*a*) by reason of the oscillating electrostatic field of the atom, or (*b*) by the possible ultimate ejection of electrons from their orbits, or (*c*) by both.

11. An impinging electromagnetic wave would incline the orbital plane, for the size of the atom is such that it could be in only one of the opposite phases of the electromagnetic wave at any given time.

12. An impinging electromagnetic ray would cause oscillation of the orbital plane, for the atom would be alternately in opposite phases of the magnetic field of the ray.

13. The shorter the wave, the faster the oscillation. Hence the lengths and the intensities of waves would determine the energy absorption and therefore the emission. The length would determine the number of oscillations of the orbital plane in a unit of time, and the intensity would determine the degree of inclination of the orbital plane. This would explain the photoelectric effect.

14. Discontinuity would occur when an electron is ejected from its orbit, as in 8.

15. The electromagnetic waves thus emitted by the electrons of any molecule (and by the molecule itself, as indicated below in 17) would impinge upon the atoms of adjacent molecules, and would cause oscillations of their orbital planes (as indicated in postulates 12 and 13). Hence the atoms and molecules are constantly giving and receiving electromagnetic waves.

16. It is assumed that electromagnetic waves travel through space (or ether) without loss of energy. Hence there is, between atoms, an endless interchange of energy without loss. The energy coming into any atom oscillates the orbital plane of that atom causing the atom to emit electromagnetic waves in mathematically direct proportion to those absorbed.

17. Every atom is constantly passing through (being bombarded by) changing magnetic fields. This requires that the tension and compression stresses between the atoms of a molecule be in constant fluctuation and that the molecule itself, as a whole, be in a condition of pulsation. This condition of pulsation is really a fluctuation of the electric and magnetic fields which hold the atoms in their positions in the molecule. *Molecular emission*, therefore, results from the fluctuation of the electrostatic forces which hold the atoms in their positions (as in a radio transmitter).

18. A Theory of the Greatest Mass can be postulated. For if the energy necessary to create a given mass is the least energy, conversely for any given amount of energy the mass must be a maximum.

A CRITICISM OF THE SCHNEIDER POSTULATES ON ATOMIC AND MOLECULAR STRUCTURE

By C. P. MITCHELL

The purpose of the following article is to suggest certain modifications of the Schneider postulates in order to show a clearer relation between the electrostatic, magnetic, and electromagnetic properties of the hydrogen atom. The postulates regarding the direction of motion of the atom have been retained, but the reason for these motions has been explained on an electrical and magnetic basis rather than a mechanical and electrical basis.

It is a much debated question at the present time whether the mass of an electron is an actual gravitational attraction or whether it is due to the electromagnetic mass. The electromagnetic mass is the apparent mass of the electron due to the kinetic energy of the magnetic field surrounding it. The mathematical theory of this is given in Jean's "Mathematical Theory of Electricity and Magnetism",[1] while some experimental evidence is given in Sommerfeld's "Atomic Structure."[2] If the mass is regarded as gravitational, it will then be necessary to determine the equations of the elliptical path resulting from motion parallel to the orbital plane, and then interpret this in terms of an electrostatic field in order to determine the electromagnetic radiation. It would seem much simpler, therefore, to treat the electron as having electromagnetic mass only, since we are chiefly interested in the radiation of energy.

Let us consider a hydrogen atom having a proton in the center and an electron revolving in a circular orbit about it, and suppose for the moment that the atom is at rest. The electron would then have a magnetic field about it at right angles to the orbit, as shown ing Figure 1 (page 87). The field intensity at any point is given in Jean's "Mathematical Theory of Electricity and Magnetism."[3]

$$F = \frac{e\ U \sin \Theta}{C\ r^2}$$

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[1] pp. 585 to 592.
[2] pp. 20 and 21.
[3] p. 514

where e = charge of an electron, 4.77×10^{-10} e. s. u.

U = velocity of the electron.

Θ = angle between tangent to orbit and line to point in question.

C = ratio of electrostatic to electromagnetic units 3×10^{10}

r = distance from electron to point.

The electron in its orbit would then be similar to a closed loop of wire carrying a current.

Now suppose that the atom moves in a direction parallel to the orbit of the electron. The proton, carrying a positive charge, would then have a field also which would at times be parallel to that of the electron. Consequently as the electron revolves, its field reacts with that of the proton as shown in Figure 2 (page 87). At the top of the orbit the electron is acted on by a force which tries to push it into the nucleus. When it is diametrically opposite to this position, it is acted on by a force which tends to push it away from the nucleus. This would, therefore, make the orbit elliptical. It is also possible that, as the electron revolves, there will be variations in the electrostatic field due to the reactions of the magnetic fields, which will cause electromagnetic waves to be emitted. The frequency of the radiation would then be a function of the speed of the electron in its orbit.

If, however, the atom moves in a direction perpendicular to the plane of the orbit, the magnetic field of the proton would be at right angles to that of the electron at all times and there would be no force exerted between them. Movement in this direction, therefore, would require less energy than that required for motion parallel to the orbit.

The postulate regarding the orbital electrons lying in a plane is readily seen from the electrical standpoint, since the magnetic fields created by electrons revolving in the same direction would tend to line up, thereby causing the electrons all to fall in the same plane.

The remaining fourteen postulates are all in accord with the new interpretations given to the first four postulates, since they depend largely on the conceptions of the motions of the atoms, and these ideas have not been changed.

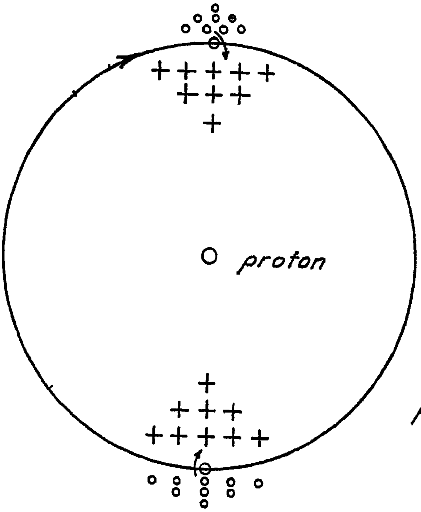


Fig 1

Magnetic Field of electron when atom is at rest

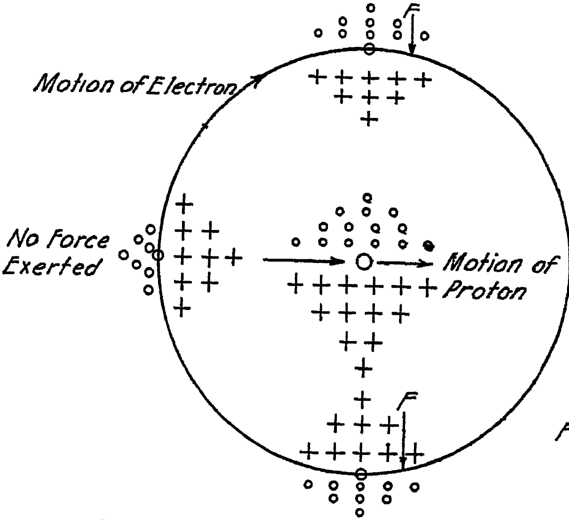


Fig 2.

Reaction of magnetic fields when atom is in motion.

ENERGY RADIATED BY AN ATOM OF HYDROGEN IN MOTION

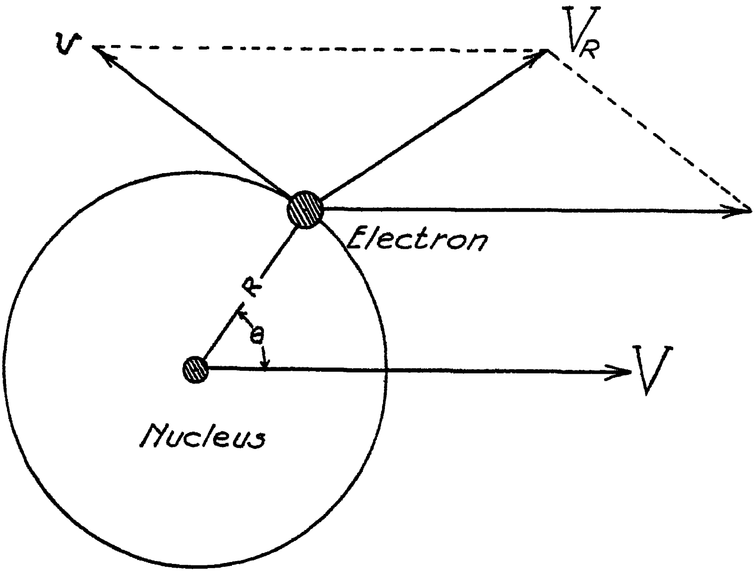
By D. A. WELLS and M. A. WELLS

According to classical theory the energy radiated per second from an accelerated charge is $\frac{2}{3}\ \frac{e^2a^2}{c^3}$ where "e" is the charge and "a" the acceleration. The electrons of any atom in motion are continually undergoing changes in speed (except when the motion is perpendicular to the plane of an orbit) which means that the electrons have a scalar acceleration.

The question has arisen as to whether this scalar acceleration of the electrons should produce radiation. It was thought of interest, therefore, to take the simple case of a hydrogen atom whose nucleus is moving with uniform velocity in a straight line and whose electron is rotating with constant angular velocity in a circular orbit about the nucleus, the plane of the orbit being parallel to the direction of motion of the nucleus, and to determine the energy radiated by the electron, *assuming that changes in speed cause radiation.*

I

SPEED OF THE ELECTRON



From the diagram above, it may be seen that

$$\text{Speed} = V_R = [(V\text{-}v \sin \Theta)^2 + (v \cos \Theta)^2]^{\frac{1}{2}}$$

and, since $\Theta = 2\pi$ ft, where f is the frequency of the electron in its orbit,

$$\begin{aligned} V_R &= [(V\text{-}v \sin 2\pi ft)^2 + (v \cos 2\pi ft)^2]^{\frac{1}{2}} \\ &= [V^2 + v^2 - 2\ Vv \sin 2\pi ft]^{\frac{1}{2}} \end{aligned}$$

Rate of Change of Speed or Scalar Acceleration of the Electron:

Now, differentiating V_R,

$$\text{Acceleration} = a = \frac{dV_R}{dt} = -\frac{2\pi fVv \cos 2\pi ft}{(V^2 + v^2 - 2\,Vv \sin 2\pi ft)^{\frac{1}{2}}}$$

II

ENERGY RADIATED BY THE ELECTRON

From the classical radiation formula, the energy radiated per second is

$$E/\text{sec.} = \frac{2}{3}\,\frac{e^2 a^2}{c^3}$$

Since this scalar acceleration is not constant but a periodic function of time (the period being that of the electron in its orbit) it is necessary to integrate over one cycle and multiply by the frequency to find the energy radiated per second.

$$\text{Now}\ \frac{dE}{dt} = \frac{2}{3}\,\frac{e^2 a^2}{c^3}$$

$$\therefore\ E/\,\text{cycle} = \frac{2}{3}\,\frac{e^2}{c^3}\int_0^{\frac{1}{f}} a^2\,dt$$

$$= \frac{2}{3}\,\frac{e^2}{c^3}\,2\pi^2 f^2\,Vv \int_0^{\frac{1}{f}} \frac{\cos^2 2\pi ft}{\frac{V^2 + v^2}{2Vv} - \sin 2\pi ft}\,dt.$$

The integral is of the form $\displaystyle\int_0^{\frac{1}{f}} \frac{\cos^2 2\pi ft}{K - \sin 2\pi ft} dt.$

where $K = \dfrac{V^2 + v^2}{2Vv}$. But $\cos^2 2\pi ft = 1 - \sin^2 2\pi ft,$

and $\dfrac{1 - \sin^2 2\pi ft}{K - \sin 2\pi ft} = \sin 2\pi ft + K - \dfrac{K^2 - 1}{K - \sin 2\pi ft}$

$$\therefore \int_0^{\frac{1}{f}} \frac{\cos^2 2\pi ft}{K - \sin 2\pi ft} dt = \int_0^{\frac{1}{f}} \sin 2\pi ft\, dt + K \int_0^{\frac{1}{f}} dt - \frac{K^2 - 1}{2\pi f} \int_0^{\frac{1}{f}} \frac{2\pi f\, dt}{K - \sin 2\pi ft}$$

A value for the last integral may be obtained by writing the integral,

$$\frac{1}{K} \int \frac{2\pi f\, dt}{1 - \dfrac{\sin 2\pi ft}{K}}$$

and expanding,

$$\frac{1}{1 - \dfrac{\sin 2\pi ft}{K}} = 1 + \frac{\sin 2\pi ft}{K} + \frac{\sin^2 2\pi ft}{K^2} + \cdots\cdots$$

which, being convergent, may be integrated term by term.

$$\therefore \int_{0}^{\frac{1}{f}} \frac{\cos^2 2\,\pi\,ft}{K - \sin 2\,\pi\,ft}dt = \left[-\,\frac{\cos 2\,\pi\,ft}{2\,\pi\,f}\right]_{0}^{\frac{1}{f}} + \Big[\,Kt\,\Big]_{0}^{\frac{1}{f}}$$

$$-\,\frac{K^2 - 1}{2\pi\,f}\left[\frac{2\,\pi\,ft}{K} - \frac{\cos 2\,\pi\,ft}{K^2} + \frac{\pi\,ft - \frac{1}{4}\sin 4\,\pi\,ft}{K^3} + \cdots\cdots\right]_{0}^{\frac{1}{f}}$$

$$= \frac{K}{f} + \frac{K^2 - 1}{2\,\pi\,f}\left(\frac{2\,\pi}{K} + \frac{\pi}{K^3} + \cdots\cdots\right)$$

$$= \frac{1}{f}\left[K - \frac{K^2 - 1}{K}\left(1 + \frac{1}{2\,K^2} + \cdots\cdots\right)\right]$$

Putting this value of the integral into the original equation and multiplying by f to get energy per second

$$E/\sec = \frac{2}{3}\,\frac{e^2}{c^3}\,2\,\pi^2\,f^2\,Vv$$

$$\left[\frac{V^2 + v^2}{2\,Vv} - \frac{\left(\frac{(V^2 + v^2)^2}{4\,V^2\,v^2} - 1\right)}{\frac{V^2 + v^2}{2\,Vv}}\left(1 + \frac{1}{2\left(\frac{V^2 + v^2}{2\,V\,v}\right)^2} + \cdots\right)\right]$$

Since $f = \frac{v}{2\,\pi\,R}$ where R is the orbital radius of the electron.

$$E/\sec = \frac{2}{3}\,\frac{e^2}{c^3}\,\frac{Vv^3}{2R^2}$$

$$\left[\frac{V^2 + v^2}{2\,Vv} - \frac{(V^2 - v^2)^2}{2\,Vv\,(V^2 + v^2)}\left(1 + \frac{1}{\left(\frac{V^2 + v^2}{Vv}\right)^2} + \cdots\right)\right]$$

Substituting known values of $v = 2.18 \times 10^8$, $R = 0.527 \times 10^{-8}$ (K orbit) and $V = 2.6 \times 10^5$ for the hydrogen atom at 0° C.,

$E/\text{sec} = 3.25 \times 10^{-7}$ ergs/sec = energy radiated per second per atom.

Assuming that all the atoms in a gas of monatomic hydrogen at 0° C. are moving under the original conditions, the rate of radiation per gram would be

$$6.01 \times 10^{23} \times 3.25 \times 10^{-7} = 1.85 \times 10^{17} \text{ ergs/sec}$$

$$= 4.6 \times 10^{9} \text{ calories/sec} = 2.6 \times 10^{7} \text{ H. P.}$$

III

SUMMARY

1. If scalar acceleration produces radiation, the electrons of monatomic hydrogen may for the most part be rotating in orbits whose planes are perpendicular to the direction of motion of the nuclei, since electrons moving in this manner have no scalar acceleration.

2. It is possible that a hydrogen atom moving under the conditions stated in the beginning can absorb energy at the same rate that it radiates.

3. Scalar acceleration alone produces no radiations.

AN APPLICATION OF FILTERED ULTRA-VIOLET RADIATION TO STERILIZATION OF DIASTATIC ENZYME SOLUTIONS

By Robert J. Norris

Employing ultra-violet radiation to sterilize a diastatic enzyme of anticipated value to the baking industry was the object of a laboratory and semi-commercial experiment by this Institute. Data from previous experiments in this laboratory on the action of such radiation upon three other enzymes had indicated a qualitative as well as a quantitative difference in response to radiations of various wavelengths, with the bacteria under similar conditions affected to a much greater extent.

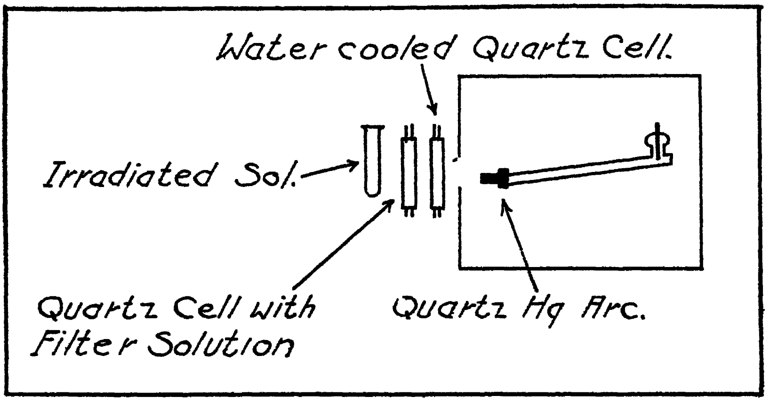
The diastatic enzyme called to our attention shortly after publication of the above mentioned results, it is believed, may serve to reduce markedly the amount of yeast, sugar, and mineral food now utilized in the process of baking. There are many difficulties presented in the production of a sterile enzyme in considerable quantity and at a final production cost low enough to make it industrially practicable in the place of common baking ingredients.

Sterilization by heat is quite impossible, and chemical agents destroy the enzyme as well as the bacteria and moreover are undesirable in food products. Of the mechanical methods, centrifugalization never completely removes bacteria; and filtration, to be effective, is too slow a process for commercial application. In the case of the diastatic enzyme, the filtration process now employed has proved entirely unsuccessful as evidenced by bacterial counts which we have made showing as high as 500,000,000 organisms per gram of dried material.

The diastatic enzyme is manufactured by allowing a pure culture of a species of Aspergillus to grow on sterile media consisting of a mash composed of various grains. Nevertheless, bacteria gain entrance and grow so much more rapidly than the mold that, at the end of the process, the filtrate containing the enzyme is heavily contaminated with bacteria. At the proper time, the mold is killed and the enzyme extracted. This filtrate is then filtered through layers of Fuller's Earth to remove bacteria, and the enzyme is precipitated.

We have made no study of the bacterial contamination in this enzyme other than to note that it is chiefly saprophytic, heat resistant, and resistant to drying. When this dried enzyme is dissolved and used in the baking mixture, bacterial growth immediately takes place. The heat of the baking process does not kill the organisms present, except on the outer surfaces. After a few days (and in moist, warm conditions, often in twenty-four hours) the bread becomes sour, and later turns green and liquefies slightly in the center of the loaf.

Encouraged by previous work with filtered ultra-violet radiations, we subjected this diastatic enzyme to radiations of the various regions of the spectrum. A Hanovia Air-cooled Quartz Mercury arc, operated at 110 volts, 5 amperes was used as a source. The diagram below indicates the arrangement of apparatus.



DIAGRAM

The quartz test tubes containing the solution to be exposed were suspended in a holder which was rotated at an uniform speed, thus insuring uniform exposure of the solution and also minimizing formation of suspended particles.

An essential factor throughout the experiment was accurate control of wave-lengths of radiation transmitted through properly concentrated filter solutions of suitable composition. When, therefore, a filter of 2540 A° is mentioned, it means that all radiations of wavelengths shorter than 2540 A° are absorbed by the filter and all longer ones are transmitted. The absorption bands of the visible and infrared radiations have not been considered. By exposing solutions with filters transmitting nearly all the far ultra-violet, and then serially changing until the last one transmits only the near ultra-violet, it is possible to study the effect of certain spectral regions and still maintain a high intensity.

I

SENSITIVITY OF DIASTASE

The first essential was to know the effect of various portions of the ultra-violet spectrum on this enzyme.

A ½% solution of this diastase was prepared in distilled water. Three cubic centimeters were placed in sterile quartz test tubes and irradiated through the filter cup containing a solution which allowed no rays shorter than 2250 A° to pass. A similar procedure was employed with each of the filter solutions until the region was covered. The time of exposure was fifteen minutes at 10 cm. from the arc. Reference to Table I (below) will show that there is an interval of approximately 100 A° between filters and that the various points determined may vary by that amount.

After the twelve exposures had been made, 1 c.c. was removed from each tube and added to 50 c c. of 1% starch solution. Digestion was continued for one hour at 38° C and the reducing sugar was determined by the method of Shaffer and Hartman[1]. The amount of sugar formed by 1 c.c. of the non-irradiated enzyme was taken as 100% enzymic activity. The percentage of active enzyme in the irradiated material was taken as directly proportional to the amount of reducing sugar formed. Table I and Figure I (page 101) show effects produced by various portions of the ultra-violet spectrum upon the activity of this enzyme.

TABLE I

Time of Exposure = 15 min.

Distance = 10 cm.

Tube	Filter λ	mgs. Maltose	% Enzymic Activity
1	Control	93	100
2	3450	93	100
3	3170	92	99
4	3050	92	99
5	2960	92	99
6	2880	90	97
7	2800	89	96
8	2760	81	89
9	2650	74	80
10	2540	68	73
11	2450	63	67
12	2350	66	71
13	2250	65	70

---

[1] Shaffer, P. A and Hartman, A. F , J. Biol Chem XLV, 365, (1920-1921).

II

BACTERIAL SENSITIVITY

The second essential was to make parallel tests for bacterial sensitivity in the ultra-violet spectrum.

A fresh ½% solution of the enzyme was prepared just before the bacterial counts were made each time. Three cubic centimeters were pipetted into sterile quartz test tubes and exposed to the radiations coming through the various filters as described in the preceding section. These tubes received a fifteen minute exposure as did the tubes for enzymic activity determinations It was found very important to have the liquid in constant motion in the test tubes to prevent the bactericidal action from becoming very erratic, due probably to incomplete exposure of some organisms.

Dilutions of 1:100, 1:1000, and 1:10,000 were made and 1 c.c. of each of these was plated in nutrient agar, incubated thirty-six hours, and counts were made by averaging the 1:1000 and 1:10,000 dilutions in the case of the controls and 1:100 and 1:1000 in the case of the irradiated solutions. Figure I shows the curves for bacterial and enzymic activity plotted together. Table II shows the results obtained.

TABLE II

Exposure—15 min.

Distance—10 cm.

Source—Quartz Hg. Arc.

Tube	Filter λ	No. Organisms per c.c.	% Orginal Orgs. Living
1	Control	240,000	100
2	3450	“	100
3	3170	“	100
4	3050	“	100
5	2960	“	100
6	2880	200,000	83
7	2800	170,000	70
8	2760	Sterile	0
9	2650	“	0
10	2540	“	0
11	2350	“	0
12	2250	“	0

III

STERILIZATION AT VARIOUS WAVE-LENGTHS

Referring to Figure I, it is evident that different wave-lengths have different degrees of destructive action upon the enzyme. With this established, it became necessary to compare accurately the sterilizing efficiency of these radiations with their destructive action upon the enzyme.

A series of sterile quartz test tubes were filled with 3 c.c. of freshly prepared enzyme solution. These were revolved before a filter cell filled with a solution transmitting all wave-lengths greater than 2250 A°. The first tube received one minute exposure, the second two minutes, the third three minutes, etc. One cubic centimeter was removed from each tube and plated on nutrient agar to determine sterility. This was repeated for each filter until the whole region had been covered, thus giving the time necessary to sterilize at any wave length. The values are stated in Table III and Figure II (page 102).

TABLE III

Time Required for Complete Sterilization at Various Wave-Lengths.

Conc. Enzyme	Filter λ	Time for Complete Ster.
0.5%	2250	2 min
"	2350	2 "
"	2450	4 "
"	2540	4 "
	2650	4 "
"	2760	8 "
"	2880	52 "

IV

ENZYME AFTER STERILIZATION

The percentage of enzyme remaining after complete sterilization at various wave-lengths was next to be determined.

A ½% solution of the enzyme was therefore exposed under the same conditions before each filter for the time found necessary to sterilize it. After exposure, 1 c.c. was removed and added to 50 c.c. of 1% starch solution. Digestion was carried on for one hour at 38° C. The amount of reducing sugar was determined as previously described. Results are given in Table IV and Figure III (page 103).

TABLE IV

Percentage Active Enzyme at Complete Sterilization—Various λ

Filter	Sterilizing Exposure	mgs—Maltose	% Active Enzyme
2250	2 min.	72	82
2350	2 "	72	82
2450	4 "	69	79
2540	4 "	71	81
2650	4 "	79	90
2760	8 "	82	93
2880	52 "	70	80
Control	0	88	100

Several interesting points are shown in Table IV. The sterilization with filters transmitting 2250 A° and 2350 A° is more efficient than with those transmitting 2450 A° and 2540A°. Filters 2650 A° and 2760 A° sterilize with only a difference of 3% in enzyme concentration but 2760 A° filter requires twice the exposure of the one at 2650 A°, so a choice between these two depends upon whether one seeks conservation of enzyme or rapidity of process.

The result obtained with filter 2880 A° is interesting because, at this wave-length, little injury is done to the enzyme (see Figure I), but it also represents a point of slight bactericidal action, so that when the exposure is sufficiently prolonged to kill all the organisms, a great loss of enzyme takes place. In Figure I it is noted that at 2760 A° the curves for bacteria and enzyme are furthest apart. That this is the most favorable filter is substantiated by the data in Table IV.

V

CONCENTRATION AND TIME FOR STERILIZATION

So far the following factors have been considered: (1) Diastatic and bacterial sensitivity; (2) Time of sterilization at various wave-lengths; (3) Percentage of enzyme remaining and optimum wave length. These factors have all been determined upon a ½% solution. This, obviously, is too dilute a solution to make such a process commercially practical. Various concentrations of this enzyme, therefore, were made and the length of time required to sterilize them deter mined. The method employed was the same as that given in Section III for sterilization at various wave-lengths.

The only difficulty encountered in these high concentrations is the removal of suspended particles. Even in low concentrations the presence of suspended matter always results in incomplete sterilization of the solution. As this difficulty cannot be remedied by prolonged exposure, careful centrifugalization or filtration is necessary before radiation is applied.*

The relation between time necessary for sterilization and concentration of the solution is shown in Table V.

TABLE V

Conc. of Enzyme	Time of Sterilization
0.5%	2 min.
1.0	8 "
2.0	12 "
3.0	17 "
4.0	28 "
5.0	30 "
6.0	32 "
7.0	32 "
8.0	32 "
9.0	34 "
10.0	36 "

Figure IV (page 105) shows that when a concentration of 4% is reached, the time of sterilization remains almost constant. This is undoubtedly due to the fact that, with low concentrations, there is a great deal of penetration of the solution by the bactericidal wave-lengths. When a concentration of 4% is reached, in all probability, the sterilization becomes a pure surface effect. It is, therefore, obvious that the bacteria must be brought into the surface film to be destroyed.

Another point to be considered in regard to the present data is that the values given for times of sterilization are purely relative, and depend on many factors such as the thickness of solution exposed, the character of the solution, the distance from the lamp, and the energy distribution of the arc itself when operated at various voltages.

---

*Since these experiments were performed we have received a new supply of enzyme containing practically no insoluble material.

Reducing the thickness of the solution from 1 cm., which was used in these experiments, to a surface film would enormously reduce the time required for sterilization.

The relative destructive action of various wave-lengths upon diastase and bacteria is a constant value, independent of time of exposure, thickness of film of liquid, or other variables.

VI

SUMMARY

1. The sensitivities of bacteria and a diastatic enzyme to various portions of the ultra-violet spectrum have been studied and found to be closely similar qualitatively but to have marked differences quantitatively in the same spectral region.

2. For the filters described, those transmitting 2250 A° sterilize the enzyme solutions in one-quarter the time of those transmitting 2760 A°. From 2760 A° to those transmitting 2880 A°, the ratio is approximately 1:7.

3. The diastatic enzyme from a species of Aspergillus can be completely and most favorably sterilized by ultraviolet radiations of 2760 A°, with a loss in activity of only 7%.

4. Concentrations of enzyme solution varying from 1—10% may be completely sterilized in the absence of suspended material.

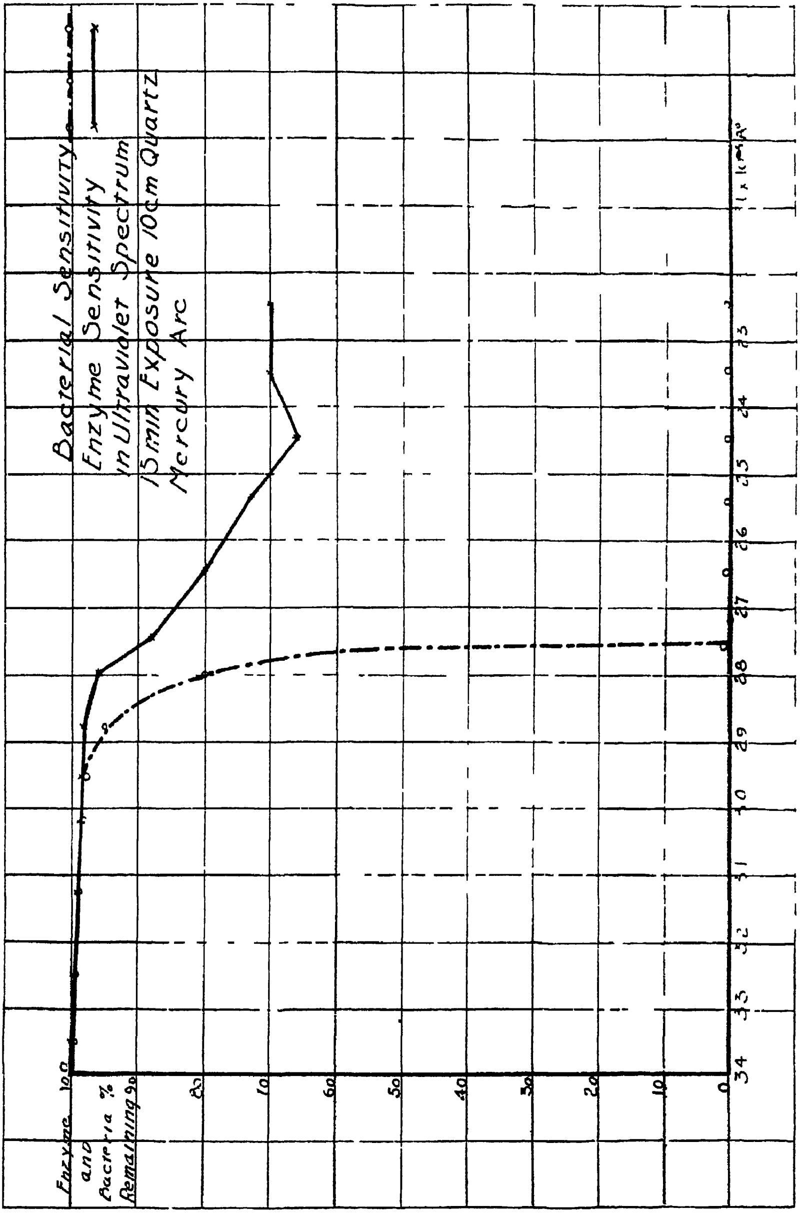

Bacterial Sensitivity
Enzyme Sensitivity
in Ultraviolet Spectrum
15 min Exposure 10cm Quartz
Mercury Arc
Enzyme and Bacteria % Remaining
100
90
80
70
60
50
40
30
20
10
0
34
33
32
31
30
29
28
27
26
25
24
23
λ x 10² A°

FIGURE I

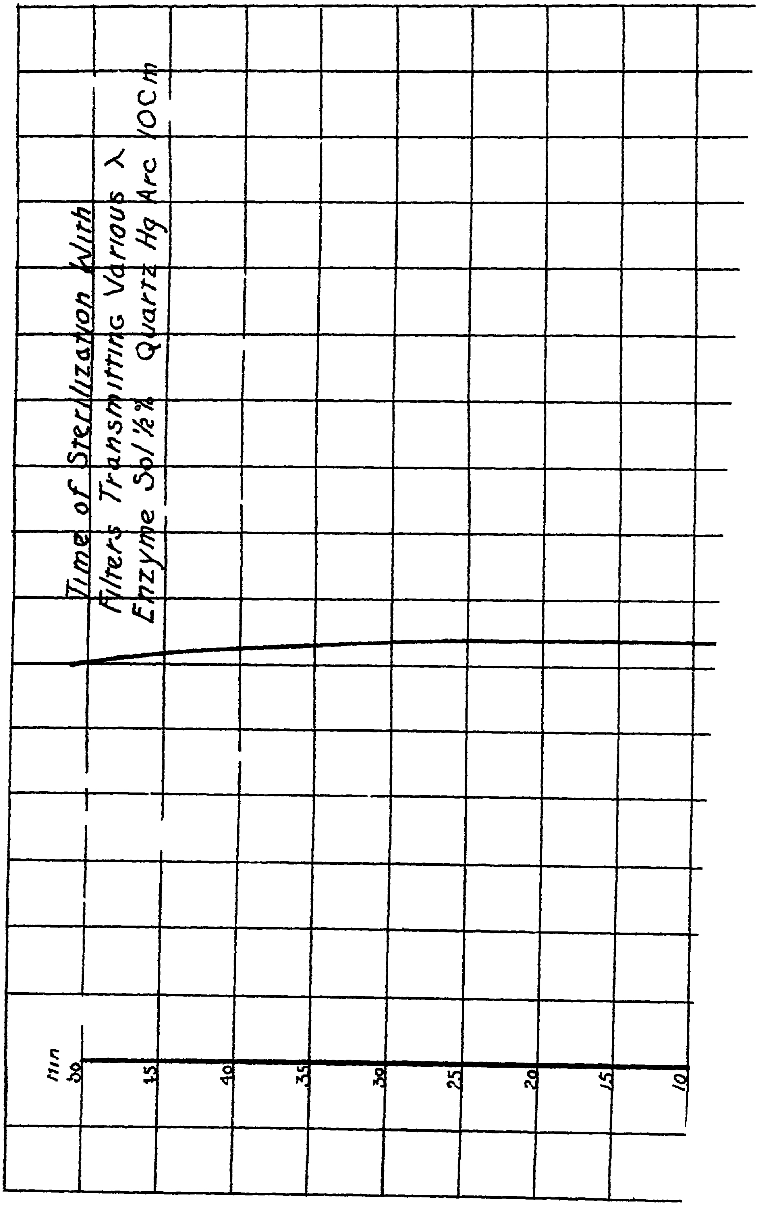

Time of Sterilization With
Filters Transmitting Various λ
Enzyme Sol ½% Quartz Hg Arc 10 Cm
min
50
45
40
35
30
25
20
15
10

FIGURE II

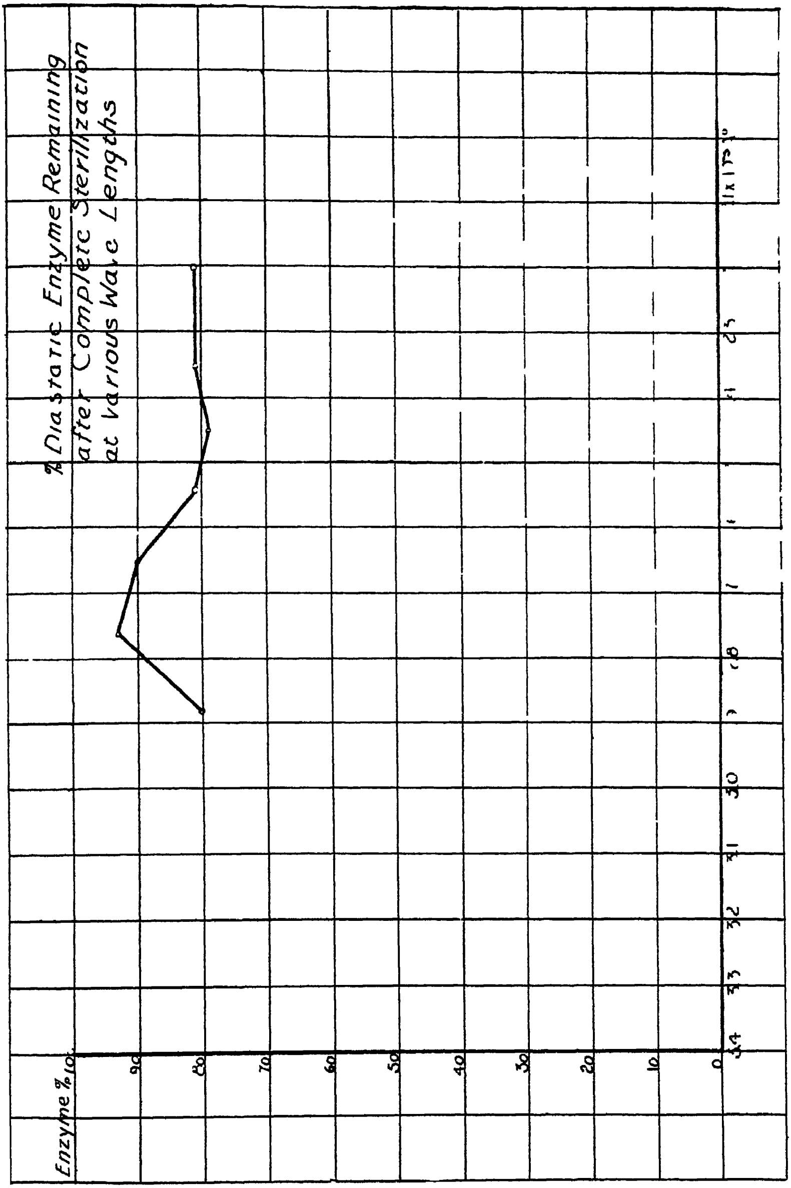

% Diastatic Enzyme Remaining
after Complete Sterilization
at Various Wave Lengths
Enzyme % 100
90
80
70
60
50
40
30
20
10
0
34
33
32
31
30
28
25

FIGURE III

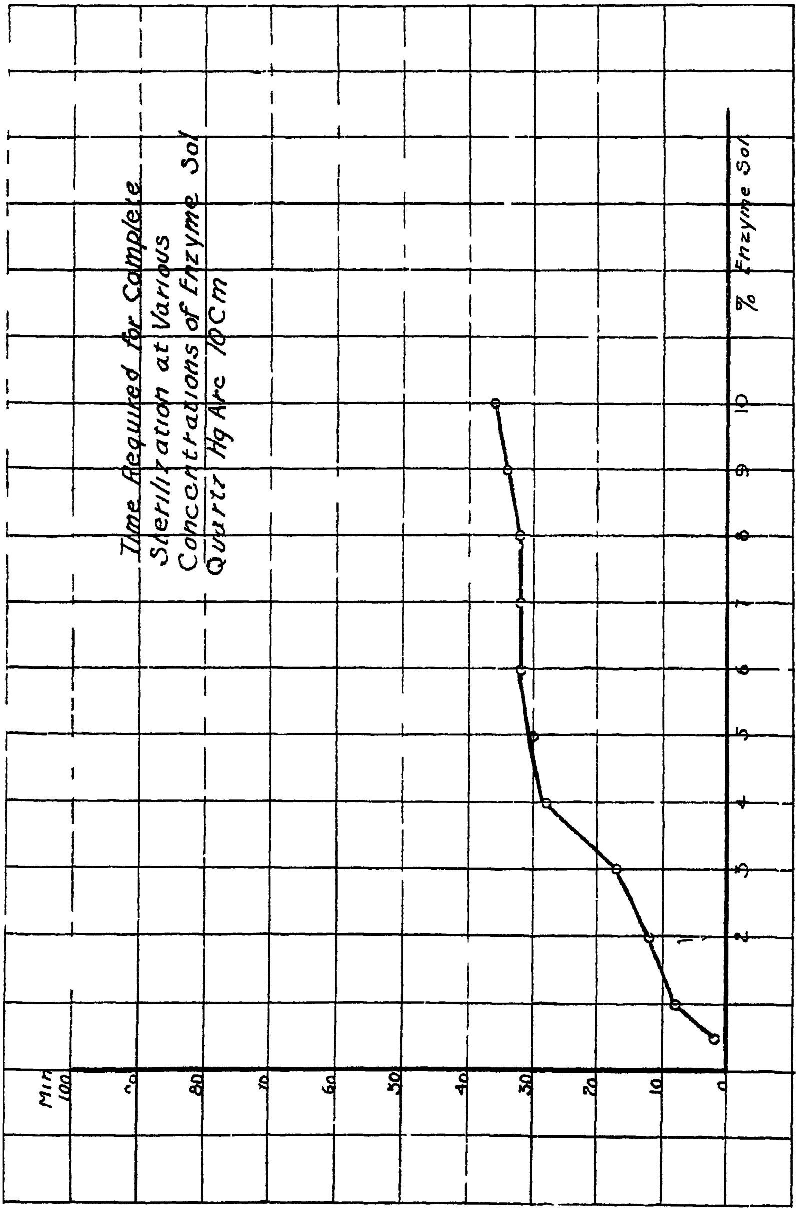

Time Required for Complete
Sterilization at Various
Concentrations of Enzyme Sol
Quartz Hg Arc 10Cm
% Enzyme Sol
1
2
3
4
5
6
7
8
9
10
Min
100
90
80
70
60
50
40
30
20
10
0

FIGURE IV

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NOTE: The above papers were received for publication October, 1929

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FOREWORD

Science has reached the point of synthesis, or, as the military men say, we are consolidating our position. Heretofore each specialized branch of science has been making its forays into the region of the unknown with little regard to the forays of the other groups. A few groups have made joint attacks, as, for example, astronomy and physics, and the results of their combined thrust into the fields of the infinitesimal and the infinite have been startling compared to the individual thrusts, for example, of zoology or botany. Medicine is joining with chemistry, and chemistry with physics, and physics with mathematics. In every case, the alliance has added greatly to the vigor of the attack, and, of course, to the amount of scientific material obtained.

The synthesis does not stop with the alliance of the sciences. Thus Huntington* makes a good case for his thesis that the life, the habits, and the customs of a people depend greatly upon their climate, their topography, and their resources; or, stated another way, psychology, philosophy, religion, art, physical attributes, and so on have their backgrounds to a large extent in mathematics and physics. The amount of radiation per year from the sun, which any country receives, has much to do with how it solves its problems in morals, art, economics, government, and all other factors which, integrated, make its civilization.

The Basic Science Research Laboratory was established in 1925 to attempt a coöperative attack of the physical sciences on questions of the most fundamental nature. The major sciences represented are mathematics, physics, chemistry, physical chemistry, bio-chemistry, and biology. Its efforts to date have been principally in developing definite relations between the physical sciences and the biological sciences. It assumes that there are two major fields of science,—the science of matter and the science of mind,—and that these two are related. Its work now is wholly in the science of matter. It plans later to add the mental sciences.

The papers presented in this booklet are all parts of larger problems. Thus the paper "The Action of Low-Velocity Electrons on Microörganisms" is additional evidence to that already published by this Laboratory on the action of the quantum theory in biological processes. Since the workers in the Laboratory pool their efforts in various problems which synthesize into larger ones, the results are group results.

HERMAN SCHNEIDER,
Director, Institute of Scientific Research.

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*Elsworth Huntington, "Civilization, Climate, and Pulse of Asia".

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THE ACTION OF LOW-VELOCITY ELECTRONS ON MICROÖRGANISMS

By D. A. WELLS

SUMMARY

1. Staphylococcus albus can be subjected to a vacuum of the order of 5×10^{-6} mm. of mercury for as long as eight hours without showing signs of killing.

2. Staphylococcus albus is killed when exposed to a beam of low-velocity electrons.

3. Under the conditions of this experiment there is a rapid rise in killing in the vicinity of 25 to 30 volts, with constant total energy, and therefore the lethal action is a function of the energy of individual electrons.

4. The per cent killed at a definite constant electronic energy is a function of the total energy of exposure.

INTRODUCTION

It has long been known that certain forms of radiant energy, such as ultra-violet light and X-rays, are destructive to various types of microörganisms. Recently it has been shown (1) that in the ultra-violet region of the spectrum there is a rather critical wave-length below which killing of microörganisms takes place, and above which there is little or no killing. This critical wave-length is approximately 2,900 Ångström units. According to the quantum theory of radiant energy, light of any wave-length has associated with it definite quanta of energy given by the equation $\epsilon = \mathbf{h}\nu$, where ϵ is the energy per quantum, $\mathbf{h}$ is Planck's constant of action and ν the frequency of the radiation. Therefore, since it is probable that the microörganisms are killed by quanta of energy greater than a definite minimum, it was thought to be of importance to investigate the action of a homogeneous beam of low-velocity electrons on microörganisms and thus attempt to determine whether the energy of moving electrons produces the same general result as that of ultra-violet light or soft X-rays.

So far as the author knows, no work of this nature has previously been done. Some rough experiments on the action of high-velocity cathode rays in air on microörganisms have been tried by Coolidge (2), but his results give no indication of the action of electrons of known energy. Again, the very high voltage which he found necessary in order to obtain a beam of electrons in air was of such value as to give each electron an energy far greater than that associated with a quantum of ultra-violet light.

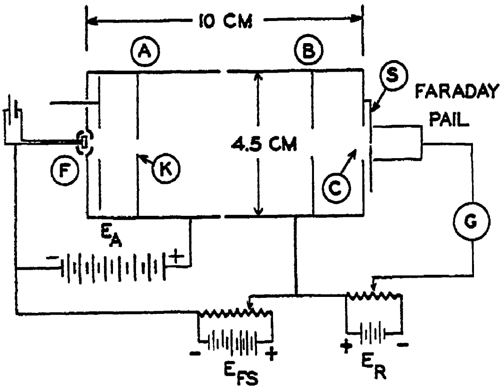


Fig. 1—First Apparatus

EXPERIMENTAL

FIRST APPARATUS

From the relation

$$\epsilon = h\nu = \frac{hc}{\lambda} = Ee$$

it is apparent that the voltage through which an electron must fall in order to acquire the same energy as that associated with a quantum of light of $\lambda = 3000\text{Å}$ is approximately 4.12, and that for shorter wave-lengths the voltage must be correspondingly greater. Thus, in order to irradiate microörganisms with electrons having energies of the same order of magnitude as that of the quanta of ultra-violet light, it was necessary to construct the apparatus in such a way as to give a homogeneous beam of electrons with velocities corresponding to 4 or more volts.

The apparatus first used for this purpose is shown in Figure 1. Electrons are accelerated, with a voltage of 90 to 135, from the platinum filament "F" (coated with oxide) to the first baffle in the brass cylinder "A," after which they pass through the second opening and are retarded by the baffles of the second brass cylinder "B." They finally arrive at the platinum slide "S," covered with a smear of the organism to be irradiated, with a net energy per unit charge approximately equal to "E_{FS}". A few of the electrons pass through a hole in the center of "S" and then enter the Faraday pail. By applying a variable retarding potential, "E_R", to the Faraday pail, a complete energy distribution curve of the electron beam can be obtained (see Figure 2). From this energy distribution curve, the size of the hole in "S," and the time of exposure, the total energy that falls on a square centimeter of surface can be calculated. This quantity will be referred to as "ρ."

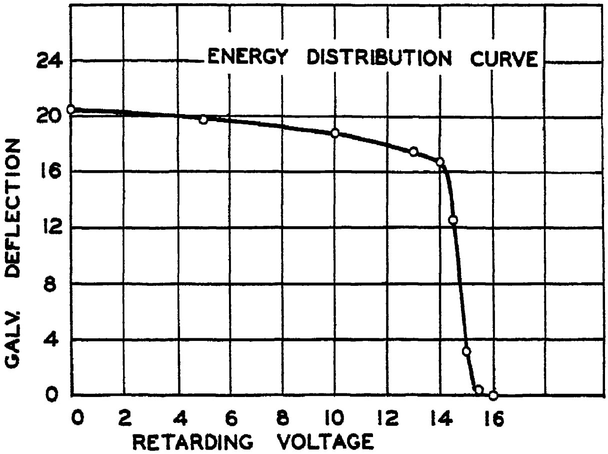


Fig. 2—Typical Energy Distribution Curve with First Apparatus

The above apparatus was mounted in a pyrex tube with connections leading out through hard wax joints. A heavy brass disc closed the end of the tube through which the slides

"S" were inserted or removed. The apparatus was mounted with its axis parallel to the earth's magnetic field, to prevent the slowly moving electrons being deflected out of their course. It was found that the wax joints held excellently, and that a very homogeneous beam of electrons could be obtained, with energy per unit charge as low as two volts.

VACUUM SYSTEM

Throughout this work the vacuum was maintained by a glass mercury condensation pump backed by a Cenco Hyvac rotary oil pump. Pressures were read with a McLeod gauge. Mercury vapor was prevented from entering the apparatus by a trap interposed between the apparatus and the pumps and gauge. This trap was kept continuously in a mush of solid CO_2 and acetone. At all times great care was exercised to see that mercury vapor did not enter the apparatus. All work was done at a vacuum of between 10^{5} and 5×10^{6} mm. of mercury. Throughout this work the pyrex containing tube was heated for some time to about 350°C before a slide was placed in the apparatus. This was done in order to reduce occluded gas and moisture on the walls of the container.

TECHNIQUE OF OBTAINING RELIABLE AND CONSTANT SOURCE OF ELECTRONS

As previously explained, an oxide-coated platinum filament was used as the source of electrons. Although the problem of obtaining the required supply of electrons from a filament may seem simple, great difficulty was encountered in making a reliable and constant source.

Flat platinum ribbon of two-ampere capacity, electrically welded to copper lead wires, was used as the filament. This was given a coat of barium and strontium oxide in the following manner. Strontium carbonate and barium carbonate were separately mixed with molten paraffine and cast into small sticks. The filament, heated to about 300°C, was stroked with the strontium stick. The temperature of the filament was then increased to about 1,000°C and kept at that value for a few seconds until all the paraffine was driven off and the carbonate changed to oxide. This was repeated with the barium, and then alternately with the strontium and barium for several times, until a heavy coat of the two oxides was deposited on the platinum filament.

This filament, mounted as shown in Figure 1, gave excellent emission at a dull red heat when first put in operation, but after a short time the emission began to decrease. At the end of a few hours the emission was practically zero, even with an excellent vacuum and all other conditions apparently favorable. The trouble was found to be due to sputtering from the first brass baffle back to the filament. Upon replacing the brass with platinum, nickel, or aluminum, no further trouble was experienced. A current of from 4 to 5 milliamperes could be drawn from a filament (only a few millimeters long) for from six to eight hours a day for several days without showing a very decided decrease in emission.

Another serious difficulty encountered was the action of the magnetic field of the filament on the slowly moving electrons. The field, due to the current in the filament and the leads, was almost completely eliminated by shaping the filament as shown in Figure 1 (a loop with a very small cross-sectional area), and by twisting together the enameled, copper lead-in wires. This made a practically non-inductive circuit.

The number of electrons entering the opening in the first baffle was found to vary considerably with the distance of the filament from the opening. The arrangement which gave a greater electronic current than any other tried is shown in Figure 1. It consists of a concave "reflector" placed directly behind the filament and insulated from its surroundings, and a slightly concave baffle in front of the filament.

In the early stages of this work it was very difficult to obtain a homogeneous beam of electrons. However, the energy distribution curves were greatly improved by following the method of Dalton and Baxter (3) and coating with soot the edges of all openings through which electrons pass. The soot apparently eliminates secondary and reflected electrons.

SELECTION AND TECHNIQUE OF HANDLING ORGANISM

Staphylococcus albus was chosen as the organism with which to work. This organism is one of the most common forms of bacteria, and is frequently found in the skin, especially at sites of inflammation. The organism is a small sphere of about 0.7μ to 0.9μ diameter, and usually occurs in grape-like clusters. It will grow either in the presence or absence of free oxygen. The reasons for selecting this organism were: (a) it is very easy to culture, (b) it grows well in definite colonies either on or under plain agar, (c) it is relatively harmless, (d) it has relatively high heat-resisting power, and (e) it has been used extensively in investigating the action of ultra-violet on microörganisms.

The technique involved in handling the organism in this work was as follows: The staphylococci were cultured in clear sterile beef broth at room temperature for twenty-four hours, after which a two-per-cent dilution of this culture in beef broth was made. A light smear of the solution was placed on the platinum slide "S." This was allowed to dry, after which the slide was placed in the apparatus and the area in front of the opening C, Figure 1 (a circle enclosing approximately 0.6 sq. cm.) was bombarded for a definite length of time with electrons of known energy. The slide was then removed from the vacuum and carefully covered with a strip of moist, solidified, nutrient agar, about one millimeter thick.

The strips were prepared in the following way: A thin layer of liquid agar was poured on a sterile glass microscope slide and allowed to solidify. A thin knife blade was then inserted under one end of the solid agar and the strip peeled from the glass. This strip, hanging from the knife, was then laid over the platinum slide. It was necessary to cover the slide in this manner in order to prevent spreading of the organisms, which certainly would have taken place had a liquid medium been poured over the slide.

The slide, thus covered, was incubated at 37°C for a period of twenty-four hours or more. The extent of the lethal action was indicated by the presence or absence of colonies on the bombarded area after incubation. Each slide was automatically its own control, since only a portion of the slide was exposed to the electrons. However, other control slides were run in the vacuum from time to time.

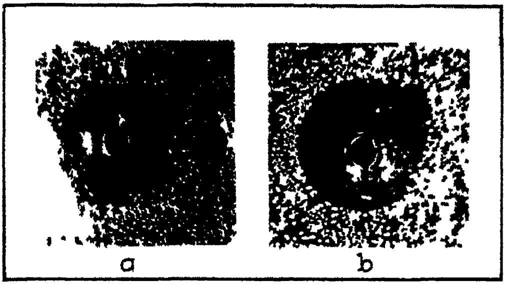


Fig. 3—Results with First Apparatus: (a) Three Volts; (b) Retarding Voltage Sufficient to Give Zero Electron Current

EFFECT OF HIGH VACUUM ON STAPHYLOCOCCUS ALBUS

It was first necessary to determine whether the organism would live in the extremely high vacuum required for this work. So far as could be found, no work had been done to determine the effect of pressures of the order of 10^{-5} or 10^{-6} mm. of mercury on microörganisms. Staphylococci on platinum slides were subjected to pressures of the order of 5×10^{-6} mm. of mercury for as long as eight hours, and after incubation as described above showed no signs of killing, the growth being the same as that on control slides.

RESULTS WITH FIRST APPARATUS

Upon irradiating the staphylococci with electrons of various voltages from ten to as low as three, it was found that there was invariably killing even with the three-volt electrons (Figure 3a). Slides were then tried with a retarding potential between "A" and "B" of such value that no electrons could strike the slide "S." Still there was complete killing over the area in front of the opening "C" (Figure 3b). This indicated that killing was probably due to a few positive rays (detectable with the galvanometer) coming from the space inside "A," or to the extremely soft X-rays produced when electrons entering the space between "A" and "B" were caused to return to the baffle "K" by the retarding field. To find the action of electrons alone these two disturbing influences had to be removed. The apparatus was therefore modified as follows:

MODIFIED OF APPARATUS

The opening "C" (Figure 1) was elongated as shown in Figure 4. The small hole in "S," and the Faraday pail, were shifted to the side, so as not to be directly opposite the opening "K." By means of a weak magnetic field, perpendicular to the axis of the apparatus, the electron beam was deflected from its normal course and made to strike the area "D" (Figure 4).

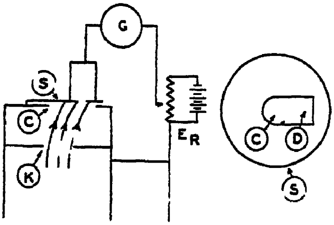


Fig. 4—Deflection of Electron Beam

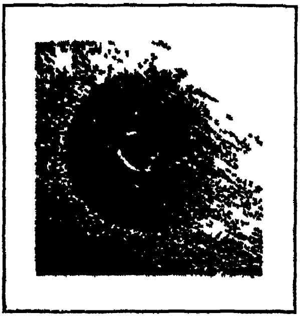


Fig 5—Test Run with Modified Apparatus

A test run was made to find if positive rays or soft X-rays were striking the area "D." A slide covered with staphylococci showed killing over the area "C" (Figure 4), as would have been expected, but none over the area "D" (Figure 5). With this arrangement the galvanometer showed no positive reading, indicating that no positive rays were striking the area "D." It was thus apparent that this arrangement excluded soft X-rays and positive rays from the region "D."

RESULTS WITH MODIFIED APPARATUS

Eleven exposures were made with electron beams of the following voltages: 6, 9.5, 9.5, 15.5, 16, 22, 28, 28, 28.5, 29.5,

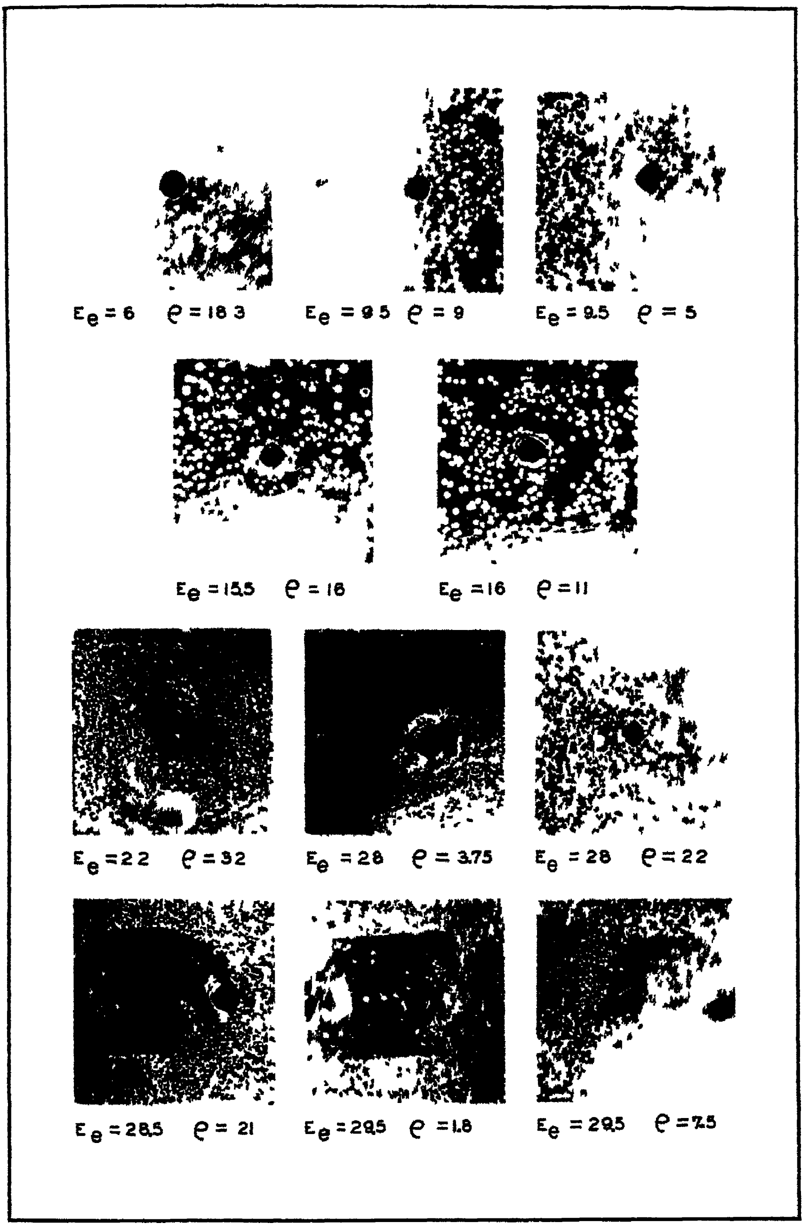


Fig. 6—Typical Results with Modified First Apparatus

29.5. The results of these exposures are shown in Figure 6.

The voltage here given is the maximum voltage in each instance of any of the electrons reaching the slide. That is to say, the voltages given represent the values at which the energy distribution curves reach the voltage axis. These values are given on the photographs, together with the total energy, "ρ," in joules per square centimeter, with which the organisms were irradiated. This energy density, "ρ," was calculated from the total area under the energy distribution curve, the area of the hole in the slide, and the time of exposure. The areas under the distribution curves were obtained with a planimeter.

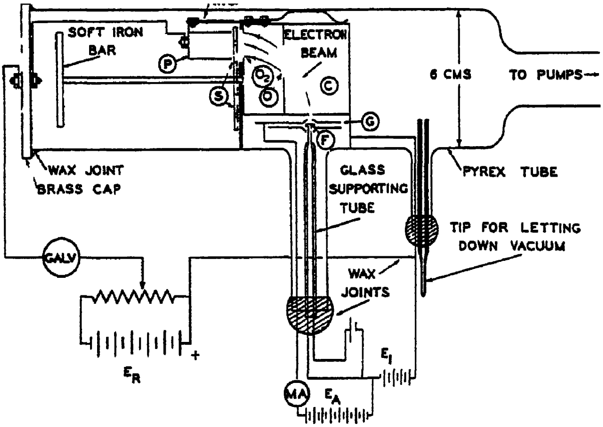


Fig. 7—Second Type of Apparatus

It is apparent that slides irradiated with electrons from 6 to 22 volts show no appreciable signs of killing. The slides at 28 volts and higher, however, show definite killing. Note that the energy density at the lower voltages is in most cases as high as—in some cases much higher than—the energy density at the higher voltages. These results therefore indicate (a) that *staphylococcus albus* is killed by electrons, and (b) that the killing is a function of the voltage through which the electrons have fallen.

After this work, it was decided to redesign the apparatus in such a way as to make doubly sure of eliminating the spurious effects previously mentioned.

SECOND TYPE OF APPARATUS

Figure 7 shows the second type of apparatus, with a diagram of the connections used. The beam of low-velocity electrons is obtained, as before, from the oxide-coated filament "F," and is accelerated to "G" by a potential "E_A" of about 90 volts. Those electrons which pass through the opening in "G" are retarded in passing to the next baffle and enter "C" with an energy per unit charge equal to "E." Thus, by varying "E," electrons of any desired voltage can be made to enter "C." After entering "C," the beam, deflected in a circular path by a magnetic field, passes through the circular openings "O_1" and "O_2", striking the platinum slide "S." The magnetic field required to deflect the beam is produced by two large, air-core solenoids.

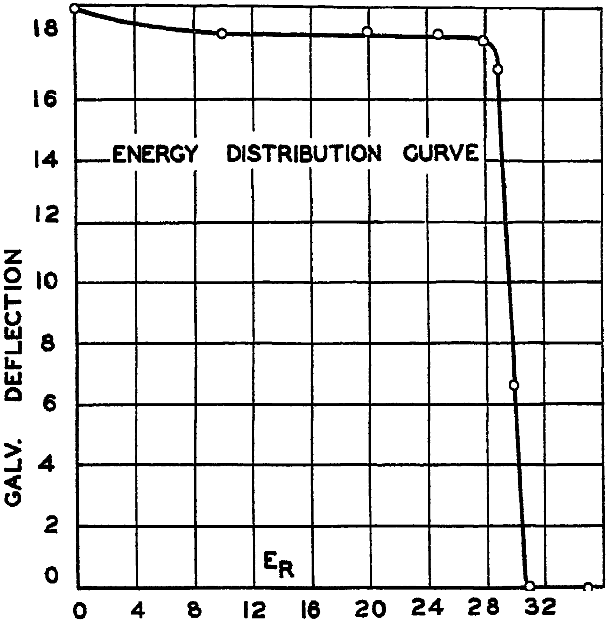


Fig. 8—Typical Energy Distribution Curve with Second Type of Apparatus

In order to pass the beam of electrons through "O_1" and "O_2" and onto the slide, the current in the solenoids was carefully adjusted while reading the galvanometer ("E_R" being zero). In this way, it was easy to adjust the magnetic field to its optimum value.

This apparatus was also arranged so that as many as four exposures could be made consecutively, without breaking the vacuum. This was accomplished by mounting four slides, equally spaced, on the face of a disc 5 cm. in diameter. The disc was mounted on an axle and so arranged that it could be turned by the action of an electromagnet on a soft iron bar attached to the opposite end of the axle. The removable bearing supporting the end of the axle containing the iron bar is not shown in the drawing, Figure 7.

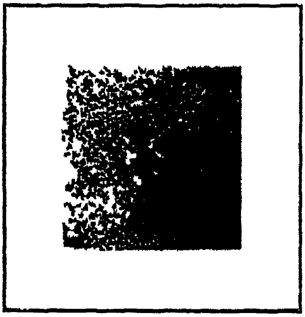
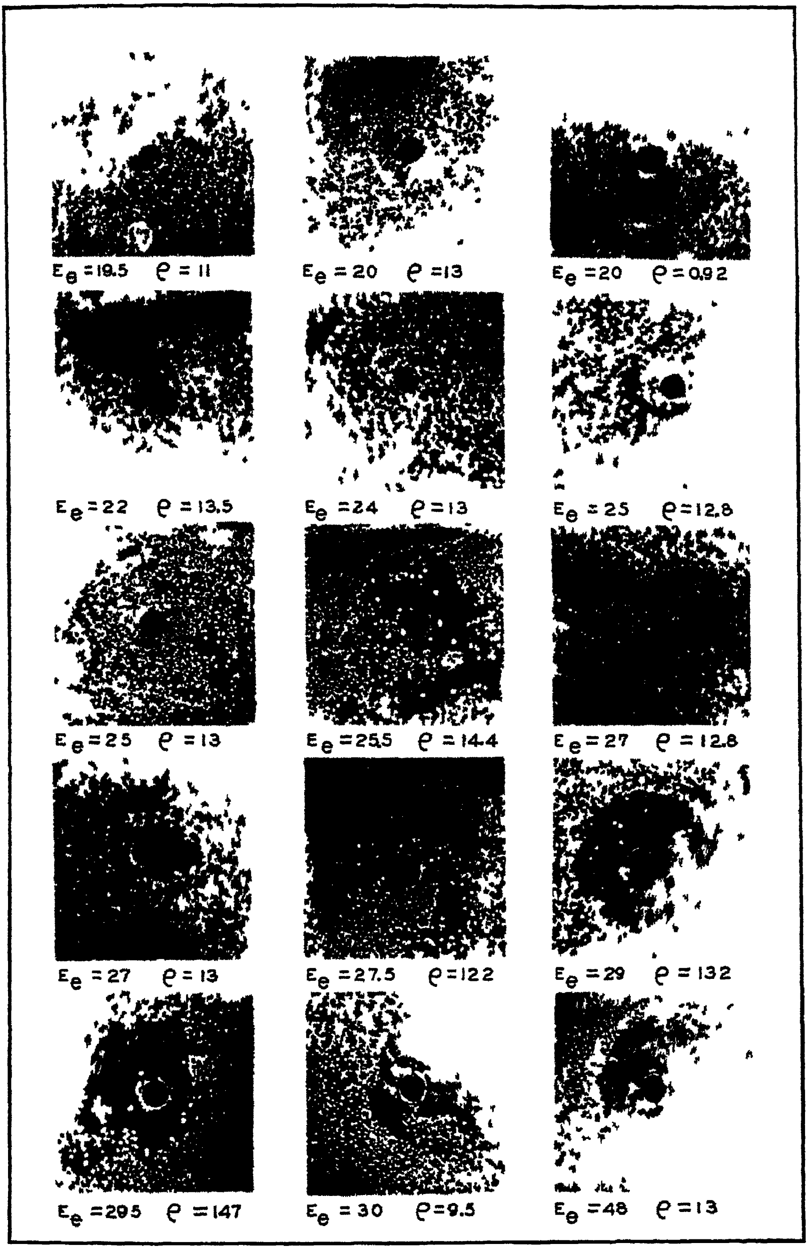


Fig 9—Test Run with Second Apparatus,
No Electron Deflection

Each slide was pierced with a hole of .028 sq. cm. area. The Faraday pail, mounted as in the first apparatus, served to determine the energy distribution of the beam. A typical energy distribution curve for this apparatus is shown in Figure 8.

The diameter of the circular opening, "O_2," is one centimeter. Therefore a spot of one centimeter diameter is irradiated with electrons. This is larger than the area that could be irradiated with the first apparatus.

With this arrangement, the action of soft X-rays produced in the vicinity of the filament should be completely eliminated, since the area to be bombarded is out of direct line with the filament and the baffles in front of the filament. The action of positive rays should also be eliminated, since any rays produced



$E_e = 19.5$ $\rho = 11$

$E_e = 20$ $\rho = 13$

$E_e = 20$ $\rho = 0.92$

$E_e = 22$ $\rho = 13.5$

$E_e = 24$ $\rho = 13$

$E_e = 25$ $\rho = 12.8$

$E_e = 25$ $\rho = 13$

$E_e = 25.5$ $\rho = 14.4$

$E_e = 27$ $\rho = 12.8$

$E_e = 27$ $\rho = 13$

$E_e = 27.5$ $\rho = 122$

$E_e = 29$ $\rho = 132$

$E_e = 295$ $\rho = 147$

$E_e = 30$ $\rho = 9.5$

$E_e = 48$ $\rho = 13$

Fig. 10—Typical Results with Second Apparatus

between "G" and the next baffles would move forward with a slight deflection opposite to that of the electrons. The result of a test run of three hours with 31-volt electrons entering "C," but not deflected to "S," is shown in Figure 9. The slide shows no killing whatever.

RESULTS WITH SECOND TYPE OF APPARATUS

Eighteen exposures were made under these conditions with electrons ranging in voltage from 19.5 to 31.5, and one exposure at 48 volts. Photographs of the slides after incubation are shown in Figure 10. It will be seen that exposures made with electrons of 19.5, 20, 22, and 24 volts show no appreciable killing, while exposures at 25 volts and higher show definite killing. It is important to note that the exposures at the lower voltages were made with as great, and in some cases greater, total energy per square centimeter than at the higher voltages, yet they show no killing. These results confirm those obtained

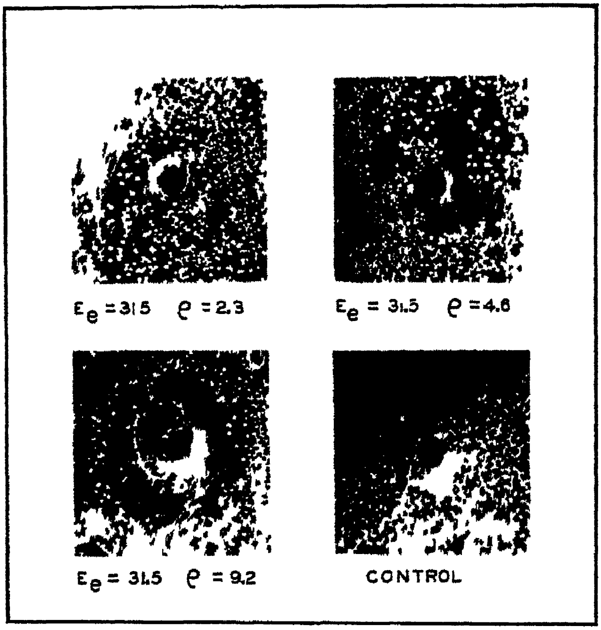

$E_e = 31.5$ $\rho = 2.3$
$E_e = 31.5$ $\rho = 4.6$
$E_e = 31.5$ $\rho = 9.2$
CONTROL

Fig. 11—Killing as a Function of Total Energy

with the first apparatus, indicating that this type of microörganism is killed by electrons, and that the killing is a function of the energy of the individual electron.

To show definitely whether the killing was a function of the total energy with which the organisms were irradiated, a series of three slides was exposed, at a constant voltage of 31.5, to total energies in the ratio of 1:2:4. A control slide was kept in the apparatus at the same time. These four slides are shown in Figure 11. It is apparent that there was a definite increase in the per cent killing with increase in total energy of exposure, which indicates that the per cent killing is a function of the total energy of bombardment

Whether the organism would ever be killed (under the conditions of this experiment) with electrons of less than 25 volts, by increasing the total energy of irradiation, is a question which can only be settled by more data. The answer depends largely on the mechanism by which the electron does the killing.

CONCLUSIONS

Although no attempt has been made to deduce quantitative relations, the results indicate that, under the conditions of the experiment, there is a rather rapid rise in the percentage of killing within the range of 25 to 30 volts with total energy constant. On the other hand, with ultra-violet light there is a rapid rise in killing in the vicinity of from 3,000 to 2,600 Å. U., equivalent to 4.12 to 4.75 volts. Again, according to Coblenz and Fulton (4), the minimum energy to kill a bacterium of *B. Coli* with ultra-violet light in the region of 1,700 to 2,700 Å. U., is about 2×10^{-4} ergs; and according to Ellis and Wells (5), the time required to kill staphylococci is somewhat less than that required to kill *B. Coli* at the same intensity. Thus the energy to kill a staphylococcus bacterium with ultra-violet light is probably somewhat less than 2×10^{-4} ergs. Now in order to get complete killing of staphylococci with electrons, the energy density required in the region of 25 to 30 volts is about 13×10^{5} ergs per square millimeter, and since the average exposed area of a staphylococcus bacterium is about 5×10^{-7} sq. mm., the average energy required to kill a single bacterium is approximately 0.65 ergs. This is about 3,000 times the energy necessary with ultra-violet radiations. It would thus seem that the action of electrons on staphylococci is different from that of ultra-violet radiations.

It must be remembered, however, that even though these results appear to be quite different from those obtained with ultra-violet light, the conditions of these experiments are also quite different. All the work on the action of ultra-violet has been done with the organisms in a rather moist state, frequently using a suspension, at atmospheric pressure, whereas this work was done in a very high vacuum, where the organisms are of necessity free from moisture. In order to make a direct comparison of the two actions it will be necessary, therefore, to carry out experiments with ultra-violet under the same high vacuum conditions

The destruction of microörganisms by electrons may be either a primary or secondary effect. The lethal action may be due to the direct impact of the electrons, to the radiant energy emitted when the electrons strike, or to an effect upon the platinum slide carrying the organisms. Likewise, the destruction of microörganisms by ultra-violet light may be either a primary or secondary action; for it has not been shown whether the killing is due directly to the ultra-violet radiation or to the photoelectrons ejected by the radiation. Furthermore, an answer to this problem would not tell how the killing takes place, since that ultimately leads to the question of what is involved in life itself.

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THE INCREASED BACTERICIDAL EFFECT OF INORGANIC COMPOUNDS IN THE PRESENCE OF X-RAYS[1]

By ROBERT J. NORRIS

SUMMARY

1. When bacteria are irradiated in solutions containing sodium bromide, sodium chloride, sodium iodide, barium chloride, mercuric chloride, uranium nitrate, cæsium iodide, or thorium nitrate, the resulting lethal action is very much greater than that obtained by exposing the bacteria successively to X-rays and to the salts previously irradiated with X-rays, and is a true synergistic action.

2. This action has previously been ascribed to ultra-violet fluorescence, or to emission of photoelectrons. Experiments are described which indicate that the synergistic action in the case of the above salts is not due primarily to these factors.

3. The experiments indicate that the synergistic action is independent of permanent chemical decomposition.

4. In the case of thorium salts, it has been shown that at the critical voltage for the ejection of the K electrons the resulting action is greatest, while theoretically the speed of the emitted K electrons is at a minimum at this voltage; and that at a higher voltage, at which the K electrons are emitted at much greater speed, the action is much less. A tentative explanation is based upon the theory that the toxicity of the ion is related to its ionic potential or state of ionization.

INTRODUCTION

The increased action of certain materials on living matter in the presence of X-rays has been described by several experimenters, most of whom have considered ultra-violet fluorescence the cause of the action. The purpose of this research was to study the synergistic action of additional materials, and to reach, if possible, some conclusions regarding its explanation.

H. S. Newcomber (1) has called attention to the increased bactericidal effect obtained by the use of fluorite containers in the X-ray irradiation of typhoid bacilli. Ghilarducci and Milani (2) have reported a greatly increased effect of Roentgen rays

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[1] Assisted by a grant from the National Research Council.

upon paramecia, ciliated epithelium, bacteria, tumors, and invertase, in the presence of fluorescent dyes and stains. W. M. Baldwin (3) has found a similar result, using paramecia. Ellinger and Gans (4), who found thorium salts to enhance greatly the action of X-rays, ascribed this action to the increased electron emission from the metal ion. Anderson (5) has found that animal tumors are more susceptible to irradiation when the animals are on a high salt diet. Peacock and Whitby (6) have attempted to show that the increased effect they have obtained by irradiating bacteria in the presence of calcium phosphotungstate is due to fluorescent light in the ultra-violet portion of the spectrum. Finally, in a previous report from this laboratory (7) some experiments on the action of cæsium iodide and X-rays are reported which show a greatly increased action of cæsium iodide on transplantable tumors in the presence of X-rays.

EXPERIMENTAL

In order to distinguish carefully between true synergistic action and the summation of the individual toxicities of the X-rays and salts, controls were used in which the bacteria received exactly the same exposure to X-rays and to the salt solution as in the experimental samples, the only difference being that in the experimental samples the bacteria were exposed to the X-rays in the presence of the salts.

Three ordinary culture tubes were used for the solutions in each experiment. Tubes 1 and 2 contained the control solutions, and tube 3 the experimental suspension. In tube 1 there was bacterial suspension only; in tube 2, salt solution only; and in tube 3, a mixture of salt solution and bacterial suspension equivalent to the combined contents of tubes 1 and 2. The organism used was a twenty-four-hour broth culture of *B. Coli.*

The tubes were X-rayed simultaneously for the same period of time, at 150 kilovolts, 19 inches target distance, using a water-cooled Coolidge tube operated from a mechanically rectified, high-voltage supply.[1] After irradiation, and a specified time after mixing the bacterial suspension and salt solution in tube 3, one cc. of the contents of this experimental tube was added to melted agar at 42°C and plated. Immediately after irradiation, one cc. of the contents of tube 1 was mixed with one cc. of the contents of tube 2. This control mixture was allowed to stand for the same time that the bacterial suspension and salt solution had been in contact in tube 3. One cc. of this mixture was then added to melted agar at 42°C, and plated. The experimental and control plates were incubated for 36 hours. Colony counts were made at the end of that time and compared with counts of plates made up in a similar manner from pure bacterial suspensions.

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[1] This equipment was very kindly furnished by the Kelley-Koett Manufacturing Company, of Covington, Kentucky.

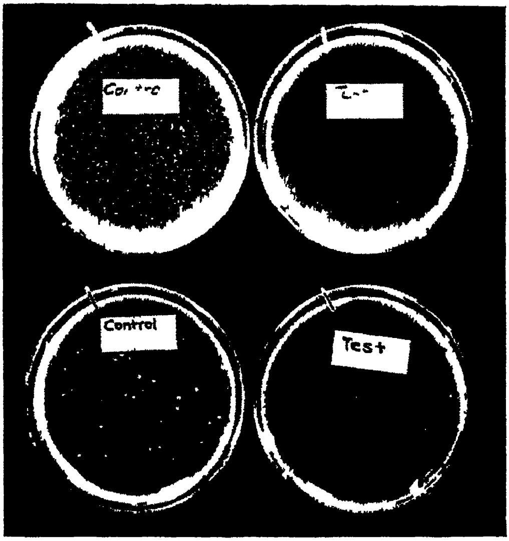


Fig. 1—Increased Toxicity of Sodium Bromide in the Presence of X-Rays Concentration 1 to 100,000

This method of experimentation eliminated from the results such factors as toxicity of the X-rays alone, toxicity of the salt alone, toxic effect of permanent decomposition of the salt (if any), and effects of fluctuations in the tube current and voltage.

SYNERGISTIC ACTION OF VARIOUS SALTS

Sodium Bromide—The early experiments with this salt showed a decided synergistic action, even in concentrations as low as 0.7 molar. At this concentration there appeared to be an increased toxicity of about twenty-five per cent as compared with the control plates. At one molar concentration the action was increased to about fifty per cent. This same percentage of increased toxicity was maintained at two molar concentration, at which about half the control plate organisms and all the experimental plate organisms were killed. Repeated experiments with concentrations less than two molar gave somewhat varied results, but the action was fairly constant with concentrations of two molar or above (Figures 1 and 2). The effect of various concentrations is shown in Figure 3.

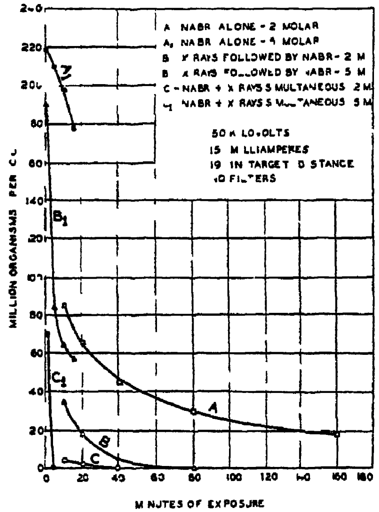


Fig. 2—Increase of Toxicity of Sodium Bromide in the Presence of X-Rays

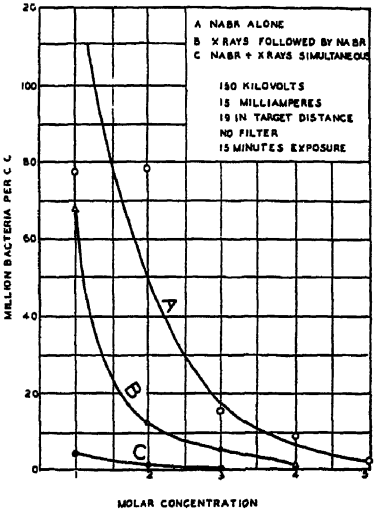


Fig. 3—Relation of Concentration to Synergistic Action of Sodium Bromide

Sodium Chloride—showed a maximum increase in toxicity of about twenty-five percent in the presence of X-rays. Its initial toxicity is, however, somewhat less than that of sodium bromide.

Sodium Iodide—which is normally more toxic than either

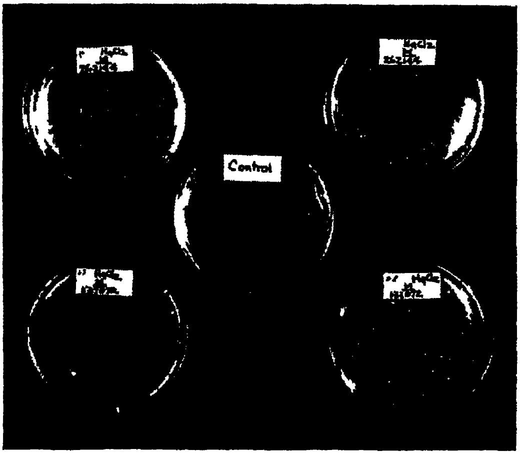


Fig. 4—Increased Toxicity of Mecuric Chloride in the Presence of X-Rays Various Concentrations

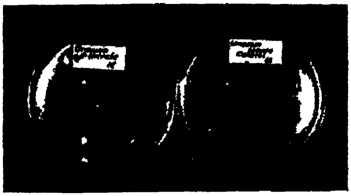


Fig. 5—Synergistic Action Uranium Nitrate, M/535,000: Left, Test; Right, Control

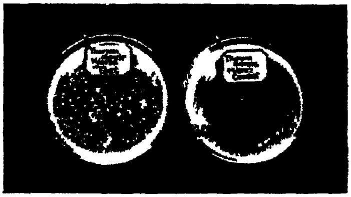


Fig. 6—Synergistic Action Thorium Nitrate, M 8192: Left, Test; Right, Control

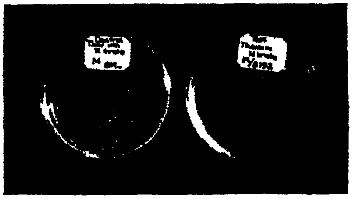


Fig. 7—Synergistic Action Thorium Nitrate, M 8192: Left, Control; Right, Test

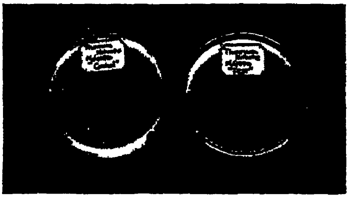


Fig. 8—Synergistic Action Thorium Nitrate, M, 4096: Left, Control; Right Test

the chloride or bromide, showed an increase in toxicity of seventy to eighty per cent. Its high initial toxicity makes it less desirable than the bromide, however.

Barium Chloride—Studies of the toxicity of this salt show that it is actively bactericidal in concentrations as low as 0.03 molar, at which concentration it is capable of reducing the number of living bacteria in an average suspension to 50 per cent of the original value in thirty minutes. There is marked increase in this bactericidal energy in the presence of X-rays. The precipitate formed in the medium prevented photographs of the results being obtained.

Mercuric Chloride and Uranium Nitrate—Experiments with these salts gave similar results, although the action was more sharply limited to certain concentrations (Figures 4 and 5).

Thorium Nitrate—As stated before, Ellinger and Gans (4) found this salt to have increased bactericidal effect in the presence of X-rays. They ascribed this action to an increase in electron emission. The technique used in these experiments was unlike that of Ellinger and Gans, but the results showed considerable synergistic action (Figures 6, 7, and 8).

Cæsium Iodide—Experiments indicating a synergistic action of this salt with X-rays on animal tumors were reported in a previous paper by G. Sperti and the author (7).

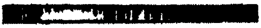
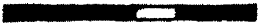
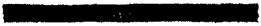
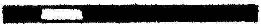
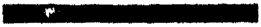
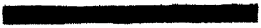
FLUORESCENCE EXPERIMENTS

It has been stated earlier in this paper that the majority of workers have attributed to ultra-violet fluorescence the synergistic action of various materials in the presence of X-rays. The author believed it advisable to investigate this possibility experimentally. Perrine (8) and Newcomber (9) have studied the spectral distribution of the fluorescent light emitted by various salts and compounds, but they have not tried the bactericidal action of these fluorescent radiations. The following experiments were not intended to be complete in the sense of considering many compounds, but were conducted for the purpose of gaining some idea of the intensity and bactericidal action of the ultra-violet fluorescent radiations from compounds used by the author in his investigations of synergistic action.

Targets were prepared by coating thin sheets of mica with the powdered material, held in place with shellac. Each target was placed with the powdered surface directly in front of the slit of a small quartz spectrograph and exposed to X-rays, with all visible light excluded. The tube was operated at 50 kilovolts, 10 milliamperes, 19 inches target distance, and ex-

TABLE I

Ultra-violet Fluorescence of Various Salts in Crystalline State

SALT	EXPOSURE	RESULTS
Hg. Arc for comparison	2 Sec.	. . 
NaCl.	2 Hrs.	Positive 
NaBr.	2 Hrs.	Positive 
CsCl.	2 Hrs.	Positive 
CsBr.	2 Hrs.	Positive 
CsI.	2 Hrs.	Positive 
CsNO$_3$	2 Hrs.	No fluorescence
Cs Alum	2 Hrs.	No fluorescence
CsCl$_3$C$_2$O$_2$	2 Hrs.	No fluorescence
LiCl	2 Hrs.	Very faint fluorescence
LiI	2 Hrs.	Very faint fluorescence
KCl.	2 Hrs.	No fluorescence
KI.	2 Hrs.	Very faint—4400–4700A.
BaPhosp-Tungst.	2 Hrs.	No fluorescence
Ca	2 Hrs.	No fluorescence
Sr.	2 Hrs	No fluorescence
SrI$_2$	2 Hrs.	No fluorescence
CeCl$_2$	2 Hrs.	No fluorescence
Zr(NO)$_2$	2 Hrs.	No fluorescence
RbI	2 Hrs.	Very faint fluorescence
Rb. Alum	2 Hrs.	No fluorescence
NaI	2 Hrs.	Very faint fluorescence

posures from two minutes to six hours were made (the average being two hours). The results are recorded in Table I.

It was found that of the substances investigated certain alkali halides emitted the greatest intensity of fluorescent ultra-violet light. Calcium phosphotungstate exhibited no ultra-violet fluorescence. Since this salt was used by Peacock and Whitby (6) in their experiments, it seems probable that their results must be explained upon some basis other than fluorescence.

To check the bactericidal effect of the fluorescent radiation, cæsium chloride and cæsium bromide were selected, since they both fluoresce in regions which are decidedly bactericidal. Petri dishes containing solidified nutrient agar were sprayed evenly with suspensions of *B. coli.* Finely powdered cæsium bromide was placed upon a thin quartz plate, separated from the surface of the agar by a few millimeters. The whole assembly was irradiated with X-rays for from one to six hours. No decrease in the bacterial count could be noticed. The experiment was repeated with cæsium chloride and calcium phosphotungstate, with negative results. It was concluded that the fluorescence of these salts is of too low intensity to have noticeable bactericidal effect.

Since the salts used in the author's experiments on synergistic action were in solution at the time of irradiation, it was decided to investigate the fluorescence of sodium bromide and cæsium chloride in solution. Concentrated solutions of these salts were placed before the spectrograph slit and irradiated with X-rays. No fluorescence could be photographed in six hours. These results further bear out the conclusion that synergistic action is not due to ultra-violet fluorescence in the author's experiments.

EFFECT OF CRITICAL VOLTAGES ON TOXICITY

As will be discussed in more detail in a following section, it was considered possible that the increased ionization resulting from the ejection of inner electrons from the atoms or ions of a toxic salt might be the cause of increased toxicity in the presence of X-rays. These inner electrons are ejected at critical frequencies, corresponding to critical tube voltages. As the tube voltage is increased through one of these critical points the emission of electrons from a particular orbit suddenly begins, and electron ejection continues at higher voltages (although to a decreased extent as the voltage is considerably increased).

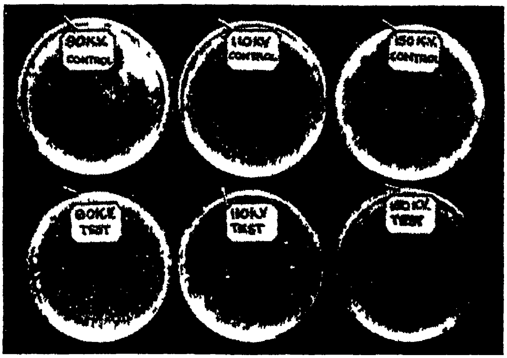


Fig. 9—Effect of Critical Voltage on Synergistic Action of Thorium Nitrate

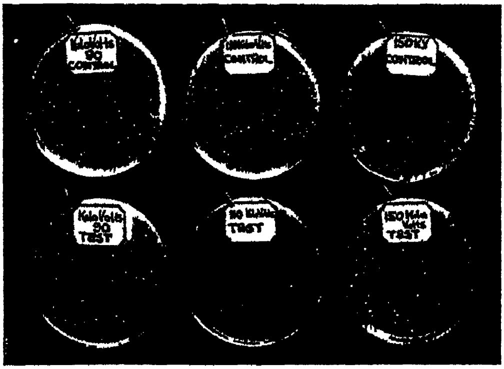


Fig 10—Plates from a Duplicate Experiment Showing Above Action

It was decided to try the synergistic action of thorium nitrate in conjunction with voltages sufficient to eject the innermost (K) electrons from thorium. By a method which will be explained in the following discussion, it is possible to calculate that the critical voltage necessary to eject the innermost (K) electrons from this element is 110,000. Voltages less than this will not eject them, and voltages considerably greater than this will have a lesser effect, as mentioned above. Thus, if voltages below 110,000, approximately 110,000, and considerably greater than 110,000 were tried, one would expect a considerably increased toxic effect at the critical voltage of 110,000, if it is true that the ejection of inner electrons increases the toxicity.

TABLE II

Lengths of Exposure for Obtaining Equal Total Energies in X-Ray Irradiation at Different Voltages

TUBE VOLTAGE	TUBE CURRENT	TIME OF EXPOSURE
150 K.V.	15 M.A.	7.5 Minutes
110 K.V.	15 M.A.	12.4 Minutes
90 K.V.	15 M.A.	18.7 Minutes

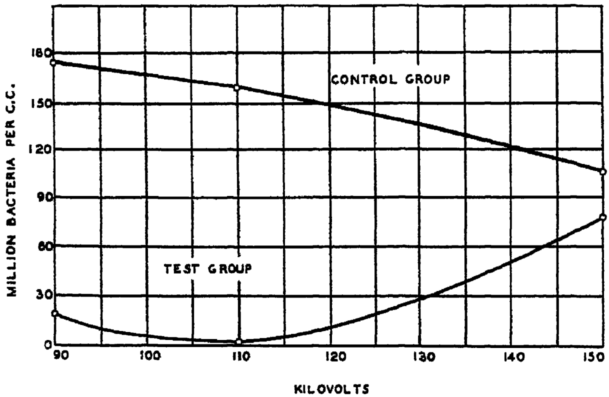


Fig. 11—Increase of Synergistic Action of Thorium Nitrate at K Voltage of Thorium

Suspensions were made up and irradiated according to the technique previously described, using thorium nitrate and

B. coli. Three tube voltages were used: 90,000, 110,000, and 150,000. Since the total energy delivered by the tube is approximately proportional to the square of the tube voltage, it was necessary to adjust the times of exposure so that the samples irradiated at the different voltages all received the same total amount of incident energy. The adjusted times of exposure, as calculated from direct ionization measurements of the X-ray intensity at different voltages, are shown in Table II.

The synergistic action decidedly increased at 110,000 (corresponding to the ejection of **K** electrons from thorium), but decreased again at the higher voltages (Figures 9, 10, 11).

THEORETICAL

Obviously the increased toxicity in the presence of X-rays must be the result of some action, primary or secondary, of the X-rays upon the materials irradiated.

When X-rays impinge on a material, a portion of the energy is reflected, a portion is transmitted through the material, and a portion is absorbed. The absorbed energy reappears in a variety of forms. These new forms are: scattered X-rays (from reflections inside the material), the character of which is very similar to that of the original beam; fluorescent radiations in portions of the spectrum other than the X-ray region; electrons ejected from the atoms of the material—which, when ejected at high velocities, are known as "beta rays," and part of the energy of which is converted into heat; and fluorescent X-rays, the character of which is different from that of the original beam, both in wave-length distribution and intensity. Through these effects, X-rays may bring about permanent chemical decomposition, increased formation of ions, or increased ionization potential of the ions present. One would expect one or more of these primary or secondary effects of X-rays to be responsible for the synergistic action.

THE VARIOUS EFFECTS OF X-RAYS

Scattered X-rays—Scattered X-rays, having approximately the same wave-lengths as the incident X-rays and being of considerably less intensity, would not be expected to have as much toxic effect as the primary X-ray beam. They probably cannot, therefore, account for the synergistic action.

Fluorescent Radiations Other Than X-rays—These include secondary radiations throughout the ultra-violet and visible regions. Of these radiations only those in the ultra-violet region shorter than about 3,000 Ångström units (11) are to any great extent lethal for bacteria. The author's experiments, previously described, show that the lethal ultra-violet fluorescence, at least in the case of the salts used in his experiments, is of too low intensity to account for the synergistic action.

Ejected Electrons; Beta Rays—According to modern theories the atom behaves very much like a miniature solar system, with a central nucleus about which electrons rotate in more or less fixed orbits or groups of orbits. Beginning with the innermost one and progressing outward, these orbits, or groups of orbits, are termed the "K," "L," "M," "N," etc., levels. Electrons may be ejected from their orbits either by the impacts of other electrons, by the impacts of positive particles (alpha particles), or by the absorption of sufficient quanta of energy from radiations incident on the atom. The innermost electrons, being most closely locked within the atom, require the greatest energy to remove them from the atom; the outermost electrons require the least energy.

In the case of electrons removed from the atom by radiations, the energy ϵ required to remove a given electron and the frequency ν of the incident radiation necessary to accomplish the removal are related by the following quantum equation:

$$\epsilon = \mathbf{h}\nu$$

in which **h** is the quantum constant. Thus, higher frequencies or shorter wave-lengths are required to remove inner electrons than to remove outer ones. Visible and ultra-violet light are of sufficiently short wave-length to eject outer electrons from many materials; but to remove electrons from **K** or **L** orbits, wave-lengths in the X-ray region are usually required. As the atoms become increasingly complex in going from lower to higher atomic numbers, the restraint on the **K** orbit electrons becomes greater, and harder and harder X-rays (that is, X-rays of increasingly shorter wave-length) are required.

Consider the **K** electrons of thorium (if this element is in a chemical compound, such as thorium nitrate, the same considerations will apply, since the effect of X-rays is essentially an atomic one). If X-rays incident on the thorium are progressively increased in frequency (i. e., decreased in wave-length), no ejection of **K** electrons will occur until a certain critical frequency is reached. Emission of **K** electrons will then commence and will continue at higher frequencies (shorter wave-lengths). At the critical frequency at which **K** electron emission begins, there will be a sudden increase in the absorption of X-rays by the atom (since energy is absorbed from the incident radiations in ejecting the electrons). The frequency at which this sudden jump in absorption occurs is called the "**K** absorption limit." Beyond the **K** limit the absorption will constantly decrease as the wave-length of the radiations is made shorter.

The **K** absorption limit of thorium (readily obtainable from absorption data), corresponding to the wave-length or frequency necessary to eject **K** electrons from thorium, is 0.1126Å. Substituting this wave-length in the following equation (deduced from the quantum expression above),

$$V = \frac{12\ 34}{\lambda}$$

in which: V = tube voltage (Kilovolts)

λ = short wave limit of emitted X-rays (A units)

the tube voltage necessary to generate X-rays of the critical wave-length to eject **K** electrons from thorium is found to be 110,000.

If the incident radiation is increased in frequency beyond the critical limit, two results follow: first, the absorption is lowered, resulting in decreased **K** electron emission; second, the excess of frequency above that required to remove the **K** electrons results in increased velocity of the ejected electrons. The velocity of ejection is given by

$$\frac{1}{2}mv^2 = h\nu - K$$

in which **K** is the energy required to remove the **K** electron from the atom, ν is the frequency of incident radiation, **h** is the quantum constant, **m** the effective mass of an electron,[1] and **v** the velocity of emission. Thus, at the critical tube voltage electrons are ejected with approximately zero velocity. As the tube voltage is increased, their number decreases but their velocity increases until they are enabled to travel for appreciable distances as high-speed "beta rays."

The previously described experimental results indicate an increased synergistic action with thorium nitrate at the tube voltage necessary to remove **K** electrons from thorium. At a higher voltage, corresponding to an increased velocity of emitted electrons, the synergistic action is less. Apparently the removal of **K** electrons results in increased toxicity; but this can hardly be explained on the basis of the impacts of emitted electrons or beta particles (suggested by certain experimenters), since the effect is greatest at the tube voltage corresponding to emission of electrons at approximately zero velocity and is less at the higher voltage. There is another possible effect of the removal of these electrons, however, which will now be described.

Increase in Toxicity With Increased Ionization—The exact nature of the toxic action of salts is not well known. As early as 1906, however, Mathews (10) suggested the dependence of toxicity upon the tendency of ions to "change their electrical state." It seems possible that the toxicity of an ion may depend upon its ionization potential. If this is true, one would expect ions from which **K** or **L** electrons have been removed

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[1] The effective mass **m** is calculated as follows:

$$m = \frac{m_0}{\sqrt{1 - \frac{v^2}{c^2}}}$$

where: m_0 = the mass at zero velocity.
v = velocity of the electron.
c = velocity of light.

(thus enormously increasing their ionic potential) to be more toxic than ordinary ions formed in solution, in which only the outermost electrons are affected. This idea seems consistent with the experimental results, since the maximum number of **K** electrons is removed near the critical voltage at which greatest synergistic action was found in the experiment with thorium nitrate.

Fluorescent X-rays—Fluorescent X-rays are caused, according to present theories, by the falling of electrons into vacated orbits, with a corresponding emission of radiant energy. It follows from the quantum equation that the wave-length of the fluorescent X-rays is governed by the energy involved in this refilling of orbits, and therefore by the structure of the fluorescing atom. In the case of thorium, the refilling of the **K** orbit by electrons dropping from the outside would result in radiations of wave-length the same as the critical wave-length required to eject these **K** electrons. This critical wave-length is, however, that of the most effective incident beam (corresponding to 110,000 volts); and since the fluorescent X-rays are never more intense than the incident rays giving rise to them, one cannot expect them to give an increased action as compared with the direct action of the original beam on the organisms. There are other orbital changes which might give rise to softer X-radiations, but the predominent fluorescence is usually considered to be of a wave length not far removed from that of the incident radiation. It does not seem probable, therefore, that fluorescent X-rays can account for the synergistic action.

Permanent Chemical Decomposition—This is a final possibility which might explain the synergistic action. As outlined previously, however, precautions were taken in the experimental work to eliminate this factor from the results. In addition, a considerable number of experiments was run in which chemical tests were made to determine the percentage of decomposition products formed. These tests and further experiments on the toxicity of the decomposition products indicated that the toxic effect of decomposition products was negligible.

CONCLUSIONS

The experimental results described and the theoretical considerations outlined lead to the conclusion that the synergistic action is probably the result of the removal of electrons from the inner orbits (**K, L,** etc.) of the toxic ions. It seems probable that this removal of electrons results in an increased toxicity of the ion itself.

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THE COMPOSITION AND PROPERTIES OF CERTAIN RED AND YELLOW PLANT PIGMENTS

By LEONORA N. BILGER

SUMMARY

Pigment extracted from Japan red peppers is shown to differ in chemical composition from lycopin or the pigment from paprika. The bleaching effects of visible light, ultra-violet light, and soft X-rays on carotin and Japan red pepper pigment are described. Ultra-violet and visible absorption data is recorded for the above pigments in various solvents.

INTRODUCTION

An investigation of the pigments of red peppers has led to certain conclusions with regard to the composition and properties of the red coloring matter of Japan peppers. In discussing plant pigments Palmer (1) points out that "active investigation of these pigments . . . has been confined to the past fifty years." A study of Palmer's monograph shows that up to 1922 the literature contained many gaps and much conflicting material with regard to the preparation, constitution, and properties of the "carotinoid" pigments. Several factors are responsible for these recorded conflicting experimental results, such as the ease with which the pigments are bleached and the difficulty of separating the red pigment from carotin.

The author has investigated carotin and the red pigment of Japan peppers. Important papers since 1922 dealing with these substances have been published by Connell (2), Schertz (3), Kylin (4), Plummerer and Rebmann (5), and Zechmeister and Cholnoky (6).

Palmer speaks of lycopin as the "characteristic red pigment of tomatoes, red peppers, the pulp of watermelon, and a number of tropical fruits." Elsewhere in his book he states that evidence indicates that it is the chief pigment in the ripe fruit of the pepper plant, Capsicum Annum. He also mentions that the "red tomato pigment has been by far the most extensively studied of the fruit pigments of the carotinoid class, and is the only one, in fact, for which we possess at present definite chemical knowledge that it is not identical with the usual carotin and xanthophylls of the chloroplastids." The work of Willstätter, Mieg, and Escher (7) has established the facts that carotin is a hydrocarbon having the formula $C_{40}H_{56}$, and that the red tomato pigment called lycopin is a true isomer of carotin.

In view of the statements in the preceding paragraph, the work of Zechmeister and Cholnoky (6) and the present investigation bring into question whether or not the red pigment of peppers or capsicum fruits and of tomatoes should both be classified as "lycopin." Zechmeister and Cholnoky isolated the red pigment from "capsicum annum L" (Spanish pepper, paprika, or pimento), calling it "capsicum red" or "capsanthin." They concluded that this pigment has fewer carbon atoms than carotin, that it has a lower molecular weight, that it is not a hydrocarbon, and that its composition is best expressed by the formula $C_{34}H_{48}O_3$, or one very similar to it. The oxygen was obtained by difference.

EXPERIMENTAL

PREPARATION

The general method of Zechmeister and Cholnoky (6) was used for the preparation of the red pigment of Japan red pepper. Certain modifications were found to increase the yield. After saponification and before separation of the filtered petroleum ether and potassium hydroxide layers it was found that long chilling in a refrigerator caused the color of the potassium hydroxide layer to become very light. After removal of the potassium hydroxide layer, addition of portions of ether to the petroleum ether layer caused separation of much soap. When this soap was removed before washing with water, much loss of pigment was avoided.

The yield was further increased by chilling during separation of the crystals from the solvent. The final recrystallization was made by dissolving the crystals in a small amount of ether, adding petroleum ether, and chilling. The final yields, however, are not comparable, since Zechmeister and Cholnoky worked with paprika and the author with red peppers.

CHEMICAL AND PHYSICAL PROPERTIES

Immediately after preparation of the deep red crystals of pepper pigment and drying in the dark and cold, ultimate analyses were made. The results of analyses were:

I. C 74.69%, H 9.95%, O 15.36%
II. C 74.69%, H 10.26%, O 15.05%
(Oxygen by difference)

If the respective percentage compositions of lycopin (7), the pigment from paprika (8), and the pigment from Japan red pepper are now considered together, it is seen that the red pigment of capsicum fruits is not identical with the so-called lycopin of tomatoes and is not the isomer of carotin. Furthermore, the analyses show that the paprika pigment of Zechmeister and Cholnoky and the pigment from Japan red pepper are not the same. The only other way of accounting for the variety of analytical data is that the original pigments are very readily oxidized. However, all experimenters emphasize the necessity for working rapidly and using samples for analysis immediately after preparation. This leads us to assume that change in composition from the original pigment has not occurred preceding analysis.

Nellie A. Wakeman's discussion (8) of homologous series of plant pigments, or more accurately of pigments referable to homologous series, suggests that pigments from different sources, varying slightly in color and properties, may vary in chemical composition in the same small degree.

Table I shows that the physical properties of all these pigments lie close together.

Palmer states that the specific rotation of carotin in alcohol, $[\alpha]_D^{20} = -30.17°$, is recorded by Kohl, but that this property is not mentioned by other investigators. The author found no rotation for carotin solution containing 0.028 grams per liter

TABLE I

Physical Properties of Pigments

Property	*Lycopin* Data from Palmer (1)	*Carotin* Data from Palmer (1)	*Capsanthin from paprika* Data from (6) Zech. and Chol.	*Pigment from Japan red pepper*
Appearance	Dark reddish brown crystals.	Bright yellow to copper-colored crystals.	Dark carmine to brick-red crystals.	Deep red crystals.
Melting Point	168°-169° C.	Original M. P. 167.5°-168° C, but falls as pigment bleaches.	M. P. differs with method of purification 167°-168° C. from methyl alcohol, 120°-130° C. if traces of impurities are present.	167°-169° C., taken immediately after purification, 120°-121° C. after slight fading, 50°-60° C. after complete fading.
Solubilities				
Ethyl Alcohol	Little.	Little.	Moderately.	Moderately.
Methyl Alcohol	Nearly insoluble	Nearly insoluble	Moderately	Moderately
Benzene	Soluble	Soluble	Soluble	Soluble
Ether		Soluble	Soluble	Soluble
Chloroform	Soluble	Soluble	Easily soluble	Easily soluble
C Disulphide	Soluble	Soluble	Little	Easily soluble
Pet Ether	Little	Soluble	Nearly insoluble	Nearly insoluble
Water	Little	Soluble	Insoluble	Insoluble
Purification	Precipitated from chloroform by alcohol and recrystallized from petroleum ether.	Precipitated from chloroform by alcohol and recrystallized from petroleum ether	Precipitated from petroleum ether and recrystallized from carbon disulphide	Precipitated from ether by adding petroleum ether and chilling. Recrystallized from CS_2.

of carbon disulphide in a 100-mm. polariscope tube with the sodium D lines. No rotation is recorded for lycopin or for the pigment from paprika. Solutions of Japan red pepper pigment in chloroform, in alcohol, and in carbon disulphide showed no rotation in a Schmidt and Haensch polariscope using the green mercury line, 5461 Å., either before or after irradiation with ultra-violet light or soft X-rays.

BLEACHING EFFECT OF LIGHT AND X-RAYS

The dry crystals of lycopin, carotin, capsanthin, and of Japan red pepper pigment are readily bleached when exposed to air and light. Alcoholic solutions of carotin and of Japan red pepper pigment may be kept in daylight at room temperature in stoppered glass test tubes (air present) for weeks without noticeable fading, and for months in a refrigerator.

Solutions of carotin containing 0.028 grams per liter and of Japan red pepper pigment containing 0.09 grams per liter were exposed in water-cooled quartz tubes to ultra-violet light and in cellophane covered cells to soft X-rays with and without air present. In general, alcohol and chloroform solutions faded much more rapidly than carbon disulphide solutions. When the tubes contained air, alcohol and chloroform solutions completely lost their characteristic orange and red colors after three hour exposures to ultra-violet light. Carbon disulphide solutions required nine hours for complete fading. When air was excluded from the tubes, about 26 hours was required for complete loss of color in carbon disulphide solutions. In all cases of exposure to ultra-violet light, a small amount of a brown resinous material formed on the walls of the tubes.

When the filtered solutions were evaporated to small volumes and cooled, sparkling, colorless crystals separated from the solvent. These crystals are at present being investigated with a view to discovering what changes occur in the composition or structure of the pigment when irradiated with ultra-violet light.

The fading of the pigment is also brought about with soft

X-rays, but the time required is very much shorter than for ultra-violet. In these experiments, a Shearer tube with an aluminum window was used as the source of soft X-rays. It was operated at from 30,000 to 35,000 volts.

A significant fact is that the pigments may be completely decolorized, whether air is present in the tubes or completely excluded, the time required in the latter case being longer. An alcoholic solution containing 0.013 grams of Japan red pepper pigment per liter was decolorized in 14 hours when air was excluded.

ABSORPTION SPECTRA

A study of visible and ultra-violet absorption spectra of carotin and of Japan red pepper pigment was made for the purpose of comparing the bands obtained with samples before and after irradiation with ultra-violet light. Neither pigment showed bands in the visible region after irradiation with ultra-violet light. In the ultra-violet region, bands were found for irradiated carotin but not for irradiated pepper pigment. Two methods were used in the case of the visible region, first, that of visually adjusting intensities by means of a Bausch and Lomb spectro-photometer, and constructing the curves from the data obtained; second, the method of photographing absorption spectra.

From the accompanying illustrations the absorption bands of carotin and of Japan red pepper pigment may be compared and absorptive properties of the pigments before and after irradiation may be seen. It will be noted that obtaining photographs of absorption spectra of these pigments depends upon the use of specific concentrations, times of exposure, solvents, and thickness of cells.

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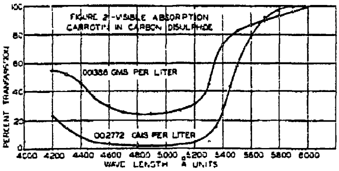
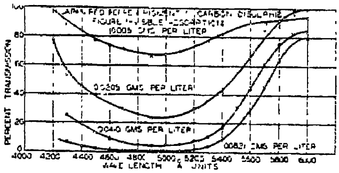
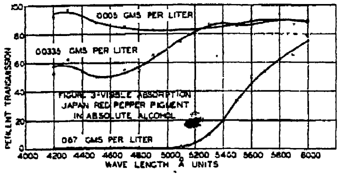
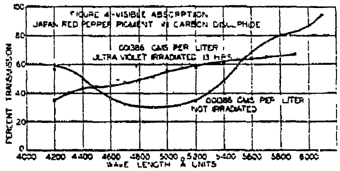
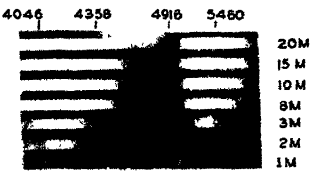




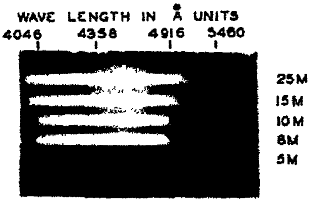




CRYSTALS OF JAPAN RED PEPPER PIGMENT

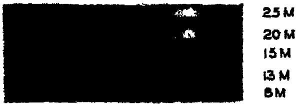


VISIBLE ABSORPTION - CAROTIN IN CS_2 - 0.0277 G. PER LITER 8 MM. CELL

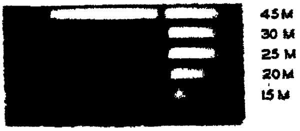


VISIBLE ABSORPTION - CAROTIN IN CS_2 - 0.0277 G. PER L. 8 MM. CELL IRRAD. WITH U.V. 8 HOURS

VISIBLE ABSORPTION - CAROTIN IN CS_2 - 0.005 G. PER LITER 50 MM. CELL



VISIBLE ABSORPTION - CAROTIN IN ALCOHOL - 0.6 G. PER LITER 20 MM. CELL



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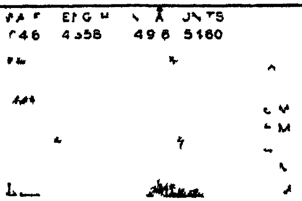
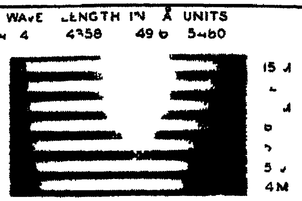


VISIBLE ABSORPTION JAP PIGMENT
IN CS$_2$ 0 005 G PER LITER
50 MM CELL



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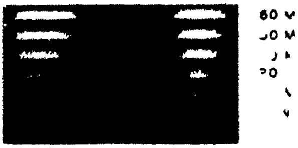
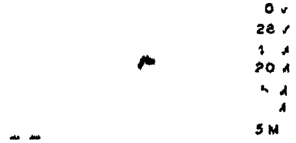
00 MM CELL



VISIBLE ABSORPTION JAP PIGMENT
IN ALCOHOL 28 G PER LITER
2 MM CELL



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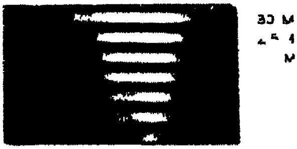
20 MM CELL



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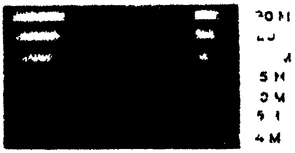
20 MM CELL



VISIBLE ABSORPTION JAP PIGMENT
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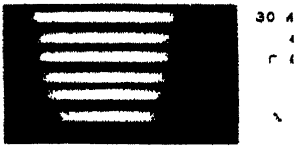
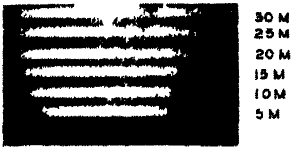
8 MM CELL



VISIBLE ABSORPTION JAP PIGMENT
IN CS$_2$ 0 08 G PER L 8 MM CELL
IRRADIATED WITH UV [illegible] 8 HOURS



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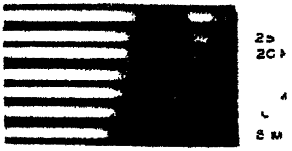
IRRADIATED WITH UV 26 HOURS



VISIBLE ABSORPTION JAP PIGMENT
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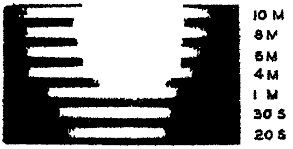
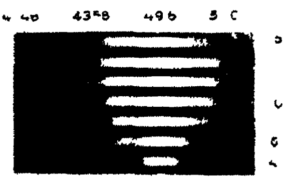
8 MM CELL



VISIBLE ABSORPTION JAP PIGMENT
IN CS$_2$ 0 04 G PER L 8MM CELL
IRRAD WITH U V 1 5 HRS IN AIR



VISIBLE ABSORPTION JAPAN PIGMENT
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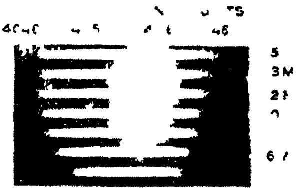

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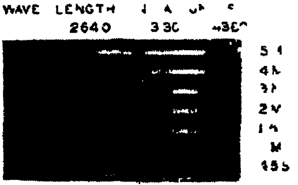
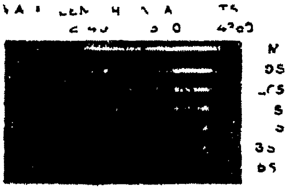
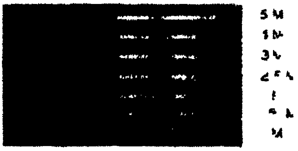


U V ABSORPTION CAROTIN
IN ALCOHOL 003 G PER L
5 MM CELL

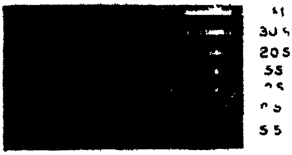


UV [illegible] CAROTIN
IN ALCOHOL [illegible] PER L
CELL



U V ABSORPTION JAPAN PIGMENT
IN ALCOHOL – 006 G PER L
13 MM CELL

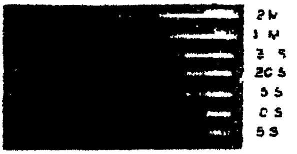
U V ABSORPTION JAPAN PIGMENT
IN ALCOHOL [illegible] G PER L
CELL



U V ABSORPTION CAROTIN
IN ALCOHOL – 008 G PER L
MM CELL

U V ABSORPTION [illegible]
IN ALCOHOL [illegible]
[illegible] MM CELL

U V ABSORPTION – CAROTIN
IN ALCOHOL 0.08 G PER L
5 MM CELL



U V ABSORPTION CAROTIN
IN ALCOHOL 008 G PER L
IRRADIATED WITH U V 23 H [illegible] CELL

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THE ANTIRACHITIC ACTIVATION OF ERGOSTEROL BY SOFT X-RAYS

By ELIZABETH SHELOW AND JOHN R. LOOFBOUROW

SUMMARY

1. Theoretical considerations show that any radiation of shorter wavelength than about 3,000 or 3,100 Angström units should be capable of activating ergosterol. This applies to radiations throughout the X-ray and gamma-ray regions. The extent of activation should not be appreciable, however. except in portions of the spectrum in which the absorption is of consequence,

2. Calculations show that the greatest X-ray absorption of ergosterol lies in the extreme soft X-ray region, and that appreciable activation should be expected in or near this region.

3. Irradiation experiments with soft X-rays are described, and absorption spectra and animal experiments indicative of the activation of ergosterol by soft X-rays are recorded.

4. The effect of X-rays on the solvent might be more pronounced than on ergosterol cr cholesterol in the case of solvents containing elements of higher atomic weight than hydrogen, carbon, or oxygen; and consideration must be given to the possibility of glass containers giving fluorescent ultraviolet radiations in the presence of X-rays.

5. The effect of soft X-rays in activating ergosterol may be a primary or secondary one.

INTRODUCTION

The antirachitic activation of ergosterol by ultra-violet light is well known, but the majority of papers on the effect of X-rays have indicated that their action is negative. Considering the X-ray activation of ergosterol from the standpoint of a theory developed by Sperti and Schneider (12) some years ago, one would expect very definite and appreciable activation of ergosterol in certain regions of the X-ray spectrum, but practically no activation in other X-ray regions.

Those who have obtained negative results in the X-ray activation of ergosterol have worked in regions in which the above theory (to be discussed in detail later) would predict no appreciable activation. The soft X-ray region, in which the theory predicts activation, had not been investigated at the time these experiments were conducted. It was decided, therefore, to study the effect of this unexplored region on ergosterol.

REVIEW OF THE LITERATURE

Hess and Weinstock (1) have reported that X-rays do not produce antirachitic changes in cholesterol, nor changes in its ultra-violet absorption spectrum.

Roffo (2) claims, on the other hand, that a 0.02 per cent solution of cholesterol in chloroform or benzene shows but traces of cholesterol by the Grigaut test after exposure for one hour to radiations from an X-ray tube, operated at 200 k. v., 4 m. a.

Reinhard and Buchwald (3), following up Roffo's work, exposed solutions of cholesterol in chloroform and in absolute alcohol to the radiations from a Coolidge tube operated at 200 k. v., 30 m. a. The solutions were sealed in glass tubes and irradiated for periods ranging from 48 minutes to 26 hours. They also exposed similar solutions to the gamma rays of radium. They state that X-rays and gamma rays produce a definite chemical change in cholesterol, as demonstrated by changes in optical rotation and ultra-violet absorption.

In a later paper, Reinhard and Tucker (4) claim that these effects are not due (at least not entirely) to action on the solvents.

Stenstrom, Lohman, and Hillstrom (5), believing that the experiments of Reinhard and Buchwald might indicate that cholesterol is antirachitically activated when exposed to X-rays in chloroform solution, carried out rat-feeding experiments with cholesterol solutions which had been sealed into glass tubes and exposed to X-rays at 180 to 200 k. v., 30 m. a., for periods of time ranging from one to fifteen hours. The experiments were negative; no antirachitic protection was found.

Sadayuki Hamano (6) found that cod liver oil, etc., affected photographic plates after irradiation with ultra-violet light, and also after irradiation with hard X-rays. He considered it possible that the similar results obtained with X-rays might indicate antirachitic activation by the latter.

Hieger (7) states that the effect of X-rays upon cholesterol in chloroform is due to the liberation of chlorine. He says that X-rays act upon cholesterol only when it is in certain chlorine-containing solvents, and that there is no action on cholesterol in benzene. Experiments with radium have led him to the same conclusions regarding the effect of gamma rays.

Steenbock (8) states that "Roentgen rays (X-rays) have a wave-length definitely below that which is necessary . . ." for the activation of ergosterol.

Hopf and Ludwig (9) found that rats died when fed upon X-rayed food, while control animals remained healthy. They obtained X-ray pictures and bone pictures which have been interpreted as indicating rickets in animals fed on the X-rayed diet.

Their work led Morrison, Peacock, and Wright (10) to determine the effect of X-rays on ergosterol previously activated with ultra-violet light. These experimenters claim that irradiation of activated ergosterol with X-rays decreases its potency. They state that X-rays have no effect in activating pro-vitamin D.

After we had completed our first series of feeding experiments, an article appeared by Delaplace and Rebière (11) stating that the irradiation of crystalline ergosterol with soft X-rays from a copper target for periods up to one hour produced changes in the absorption spectrum indicative of antirachitic activation. No statement was made as to the type of tube used, and they did not report feeding experiments.

THEORETICAL

In a paper which appeared several years ago, Sperti and Schneider (12) advanced the theory that biological and photochemical actions brought about by atomic radiations follow quantum laws. Experimental evidence was cited in the original and subsequent papers to bear out this theory.

Briefly, the theory states that, the action of short radiations on biological or chemical materials being an intra-atomic one, a critical energy level must be affected before radiations will bring about a desired change. This critical energy level is related to the frequency of the incident radiation by the quantum equation $\epsilon = h\nu$; in which ϵ is the energy per atom necessary to bring about the change, ν is the frequency of the incident radiation, and **h** is the quantum constant. Thus, in order for radiations to bring about a given chemical or biological change, they must have a frequency equal to, or exceeding, that defined by the above quantum equation. The required "critical frequency" is specific for each chemical or biological change.

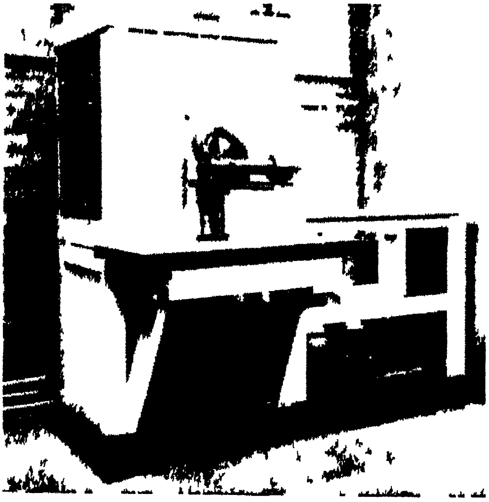

Fig. 1—Equipment

If the frequency of the incident radiation exceeds the critical one defined by the above equation, then the radiation has a sufficiently high specific energy to bring about the change. Thus, if the change begins to take place at a wave-length of, let us say, 3,000 Ångström units in the ultra-violet region, all radiations of higher frequency or shorter wave-length than this (including the extreme ultra-violet, soft X-rays, and hard X-rays) should be capable of bringing about the change.

That these shorter radiations are capable of causing the desired change does not mean, however, that they will do so efficiently. The actual amount of energy of the incident radiation transformed into biological or chemical changes depends upon the absorption by the material in question. As one progresses through the spectrum, from the critical wave-length for a given action toward shorter wave-lengths, one ordinarily finds regions of great absorption and regions of small absorption. In the regions of small absorption very little of the incident energy can be effective, whereas in the regions of great absorption much of it will be effective.

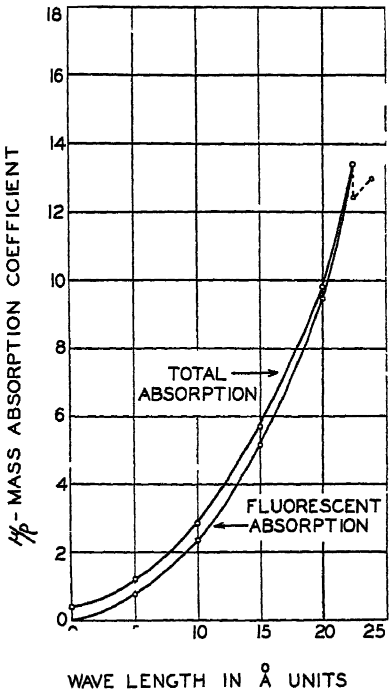

Fig. 2—X-Ray Absorption of Ergosterol as calculated from the Cube Law

In the case of ergosterol, one finds abundant evidence in the literature (which the authors have checked in their own experiments) that the activation of ergosterol begins at a wave-length of about 3,000 or 3,100 Ångström units (in the ultra-violet region), and continues for shorter wave-lengths as far, possibly, as 2,000 Ångströms. The wave-length 3,100 is, thus, on the short side of the critical wave-length for activation; and, if the theory outlined above is correct, all radiations of higher frequency or shorter wave-length than this should be capable of activating ergosterol. Radiations shorter than this critical wave-length include the entire X-ray region and the gamma rays of radium.

Whether or not X-rays and gamma rays of radium will be appreciably effective in ergosterol activation depends, however, upon their absorption by ergosterol. The absorption of X-rays by inorganic compounds follows well-known laws. It is essentially an atomic phenomenon, practically independent of the state of chemical combination of the elements. The absorption of a compound may be found by calculating the average absorption of all elements present from the absorption of each element and the percentage of each element in the compound.[1] The mass absorption coefficient (i. e., linear absorption coefficient divided by the density) of each element may be determined by means of the equation:

$$\frac{\mu}{\rho} = \frac{CN^4 \lambda^3}{A} + \frac{\sigma}{\rho}$$

in which: μ = linear absorption coefficient (absorption per unit thickness).
ρ = density of the element.
C = a constant, changing abruptly at certain wave-lengths in the spectrum, known as "absorption limits."
N = atomic number of the element.
λ = wave-length of X-rays used.
A = absolute mass of the absorbing atom.
σ = a constant representing the scattering of X-rays by the material.

From this equation it is apparent that as the wave-length decreases (in going from soft to hard X-rays) the absorption decreases in approximate proportion to the cube[1] of the wave-length, as long as C remains constant. The absorption limits at which C rises, in going toward shorter wave-lengths, end with the **K** absorption limit. For shorter wave-lengths, beyond this limit, C remains constant. The **K** limits for the constituents of ergosterol, with the tube voltages corresponding to them, are given in Table I.

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[1] Whether the absorption is independent of the state of chemical combination of the elements in the case of a complex organic compound such as ergosterol is still an open question. But we are interested here in the relative absorption in different spectral regions rather than in the absolute absorption in a given region.

TABLE I

K Limits for the Constituents of Ergosterol

ELEMENT	K LIMIT WAVE-LENGTH	CORRESPONDING TUBE VOLTAGE
Oxygen	23.34 Angströms	532
Carbon	43 250 Angströms	286
Hydrogen	911.76 Angströms	13.5

It is apparent from this table that the **K** limit of hydrogen lies in the extreme ultra-violet, while that of oxygen and that of carbon lie at the soft end of the X-ray spectrum. Therefore, throughout the X-ray and gamma ray region, from very soft rays to extreme gamma rays, the value of C remains constant for each of the constituents of ergosterol, and the absorption (exclusive of scattering, which plays no part, as far as we know, in this process) decreases in approximate proportion to the cube of the wave-length. The X-ray absorption of ergosterol rises to a maximum (corresponding to the **K** peak of oxygen) at approximately 23.5 Angstrom units (see Figure 2), corresponding to a tube voltage of 525, and decreases rapidly at higher frequencies or shorter wave-lengths.

Thus, the absorption of ergosterol in the soft X-ray region is considerable; but in the hard region, corresponding to tube voltages of, say, 75,000 to 200,000, it is extremely small. The fluorescent absorption at 6,000 volts (calculated from the cube law) is, for example, 8,000 times as great as that at 100,000 volts; or 64,000 times as great as that at 200,000 volts. One might expect, therefore, to obtain appreciable activation of ergosterol with very soft X-rays when the activation with equal energies of hard rays would be negligible.

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[1] Dr. S. J. M. Allen has shown recently that this power varies from the cube to the square in going from shorter to longer wave-lengths. The cube law is sufficiently accurate, however, for the rough comparisons to be made here.

Our own results and those of others will be discussed in the light of these theoretical considerations in a section at the end of this paper.

EXPERIMENTAL

EQUIPMENT

The apparatus used for the experiments (Figure 1) consisted of a Shearer tube fitted with an aluminum window, and in the early work a molybdenum target, in the later work a silver target. A mechanically rectified, high-voltage source[1] was used. The tube could be operated at potentials of from 5,000 to 50,000 volts.

Some difficulty was encountered in the early stages of the work because of sputtering of the window with material from the target. In a short time this would decrease the transmission of the window considerably. It was found necessary to dismantle the tube and clean the window frequently during the course of the work. Parallel experiments on the lethal action of soft X-rays on bacteria served as a check on the operation of the tube from day to day.

ERGOSTEROL SAMPLES

Ergosterol was prepared in this laboratory by the Windaus and Groszkopf (14) method. The samples used for animal experiments had optical rotations above $[\alpha]_D^{20} = -127°$. Those used for spectroscopic work had a rotation of $[\alpha]_D^{20} = -131.7°$.

From time to time, parallel experiments were conducted with ergosterol from outside sources as a check against the results obtained with our own preparations. Samples prepared by Mead

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[1] The high-voltage equipment was very kindly supplied by the Kelly-Koett Company, Covington, Kentucky.

Johnson, The Eastman Laboratory, and The Fleischmann Laboratory were used.

EXPOSURE TO X-RAYS

Samples were irradiated both in the dry, crystalline state, and in alcoholic solution (1 mg. per cc. of absolute alcohol).

In some of the experiments with dry ergosterol, a thin layer of the crystals was spread in a petri dish and exposed without a cover. In other experiments, the ergosterol was dissolved in ether, and the solution was evaporated on a glass or platinum slide so as to leave a thin film of dry ergosterol containing about one mg. per square cm. After irradiation, the slide was weighed, the ergosterol washed off with absolute alcohol, the slide reweighed, and the concentration of the irradiated solution adjusted to 1 mg. per cc. with absolute alcohol.

In early experiments in which solutions were irradiated, small petri dishes or rectangular paraffin cells filled to a depth of two or three mm., and narrow beakers filled to a depth of two cm. were used. The majority of the exposures were made with open dishes, the concentration being adjusted after irradiation by the addition of absolute alcohol. In some of the exposures the dishes were covered tightly with cellophane, to exclude air. Recently a brass cell, about two centimeters thick, with aluminum foil end windows, has been used. The cell is filled completely and tightly stoppered during irradiation.

The results were similar with all forms of containers, except that the thicker layers required longer exposures to show equal absorption changes.

The tube voltages used ranged from 10,000 to 50,000, and the times of exposure from thirty seconds to two hours. The results of these exposures are reported in the following sections.

ABSORPTION SPECTRA

Ultra-violet absorption spectra of the irradiated and nonirradiated samples were photographed with a quartz spectrograph, and a mercury arc, carbon arc, or tungsten spark source.

In the case of ultra-violet activation, the absorption changes are characterized by an increased absorption in the region 2600

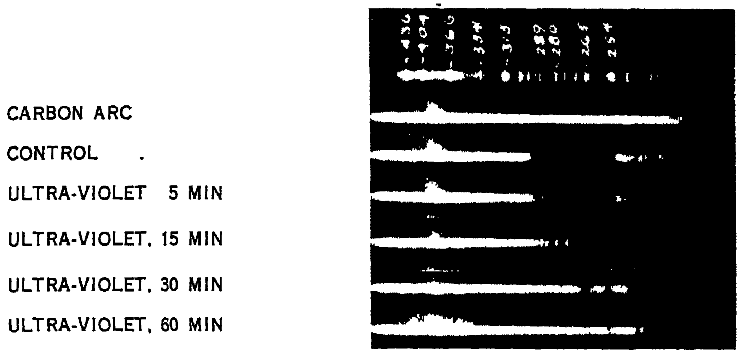
Å. to 2300 Å., and a decreased absorption in the region 3000 Å. to 2600 Å. Upon prolonged exposure, the absorption in the 2600-2300 Å. region decreases progressively. Repeating the ultra-violet irradiation experiments of Bills and Honeywell (15), we obtained absorption changes of the above type with our samples of ergosterol, corresponding closely with their results. We found, however, a variation in different samples of ergosterol. Some showed a pronounced increase in absorption in the region 2600-2300 Å. before any decrease in absorption in the region 3000-2600 Å. could be noted; others showed noticeable changes in both regions almost from the beginning; and still others showed increased absorption throughout the entire region 3000 to 2300 Å., followed, after a considerable time, by a decreased absorption extending progressively from 3000 Å. toward the short end of the spectrum (see "A" and "B", Figure 3).

In a few of our experiments with soft X-ray irradiation we obtained an increased absorption in the 2600-2300 Å region ("C", Figure 3). All other experiments consistently have shown progressively decreasing absorption in the 3000-2600 Å region, unaccompanied by any noticeable change in the 2600-2300 Å region. In this lack of change from 2600 to 2300 Å, the spectra of the majority of the soft X-ray irradiated samples differ from those of samples irradiated with ultra-violet light.

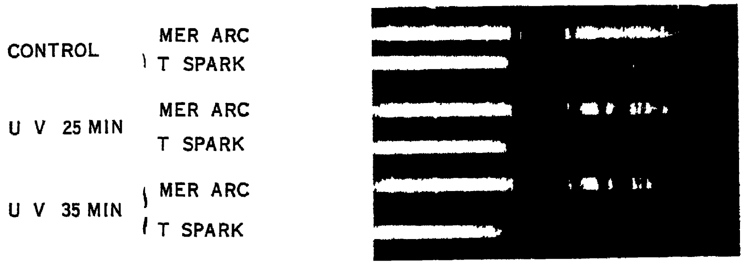
Except in a few instances, we did not obtain appreciable absorption changes at tube voltages below 30,000. This was probably due to the greater energy obtained from the tube at the higher voltages (the total X-ray energy varying as approximately the square of the tube voltage). Until recently it has been impractical to run the tube for the prolonged times necessary to compensate for the decreased energy at the lower voltages—otherwise consistent absorption changes would probably have been found at these voltages.

From absorption data, one would expect the most efficient activation to take place at tube potentials of but a few thousand volts. It is possible to study this by determining the comparative effect of equal incident energies at different tube voltages, es-

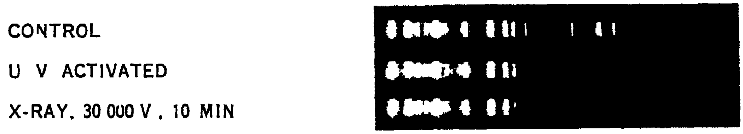
A. ULTRA-VIOLET IRRADIATED ERGOSTEROL—LABORATORY PREPARATION



B. ULTRA-VIOLET IRRADIATED ERGOSTEROL—MEAD JOHNSON PREPARATION



C. ERGOSTEROL SOFT X-RAYED IN ALCOHOL SOLUTION —FROM AN EARLY EXPERIMENT



D. SAMPLE FED TO SERIES I RATS. SEE TABLE II

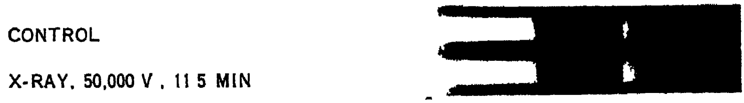


Fig. 3—Typical Absorption Spectra of Soft X-Ray Activated Ergosterol

pecially with filtered radiations. The equipment has been modified recently to permit of energy measurements, so that such experiments may be conducted.

At voltages from 30,000 to 50,000 noticeable absorption changes were obtained with exposures averaging from two to five minutes, and pronounced changes were obtained in from one to two hours.

ANIMAL EXPERIMENTS

Young albino rats weighing from 45 to 50 grams, and kept in darkened cages, were used as test animals. During the preparatory period the rats were fed only the Soames and Leigh-Clare or McCollum rickets-producing diets and distilled water, to which at intervals a small amount of iodine and potassium iodide was added. Rats that did not gain consistently were discarded. After the animals had received the rachitic diet for 25 days, an X-ray picture of the right knee joint was taken. If the pictures did not show definite rickets in the majority of animals, the rickets-producing diet was continued and pictures were taken three to five days later. Pictures of normal rats were taken for comparison, and occasionally a rat was dissected to check the interpretation of the X-ray picture. After the first group of rats had been given the Soames and Leigh-Clare diet for 32 days and the McCollum diet for 12 days, it was ready for the test period. In the second group, fed the McCollum diet alone, rickets developed quite uniformly within 25 to 30 days.

After the preparatory period, rachitic rats which were in good condition and had gained weight steadily were selected, divided into groups of four, and fed .002 mg. of ergosterol as a daily dose during these preliminary experiments. This amount is well above the minimum protective dose and could be expected to give decisive results, even though some of the material to be tested should prove only weakly antirachitic. The solutions were diluted to contain .002 mg. of ergosterol in 1 cc., and this amount was measured with a pipette and poured over a small amount of food. The alcohol was evaporated, and the

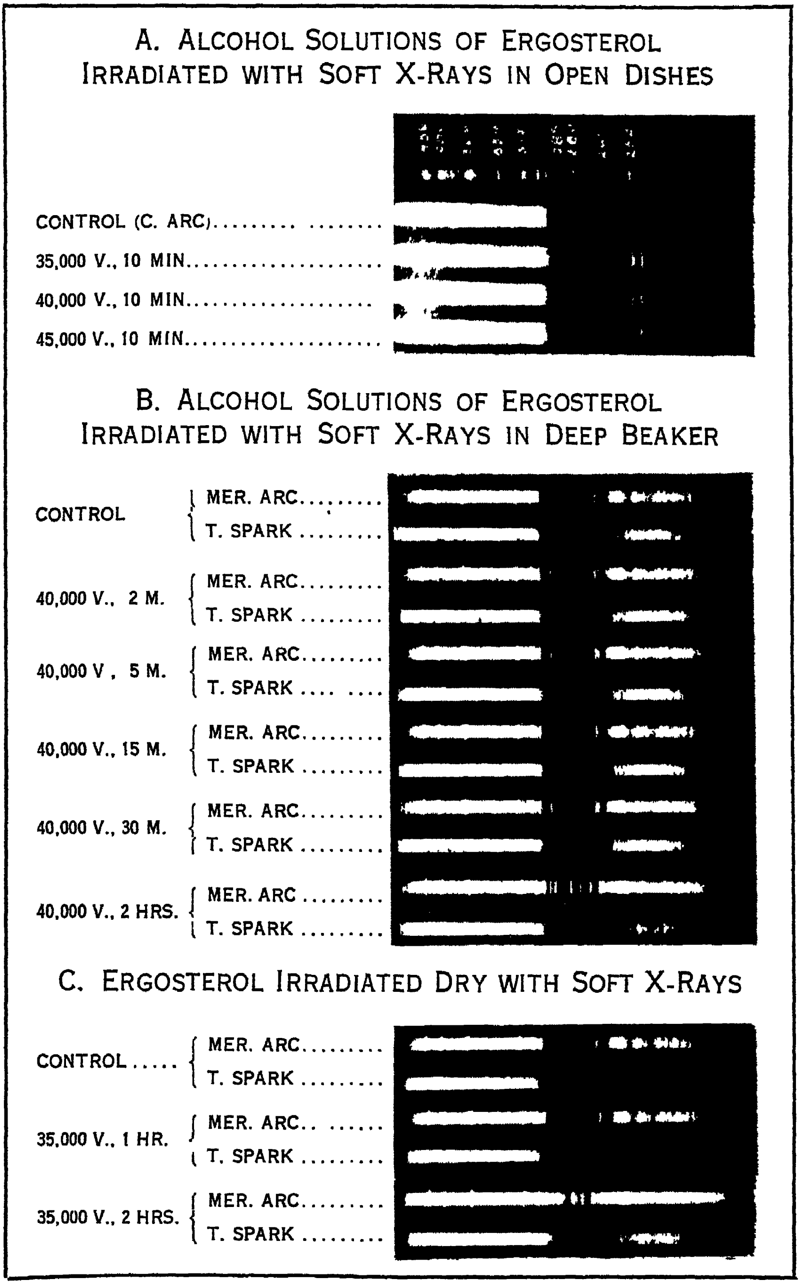


Fig. 4—Typical Absorption Spectra of Soft X-Ray Activated Ergosterol

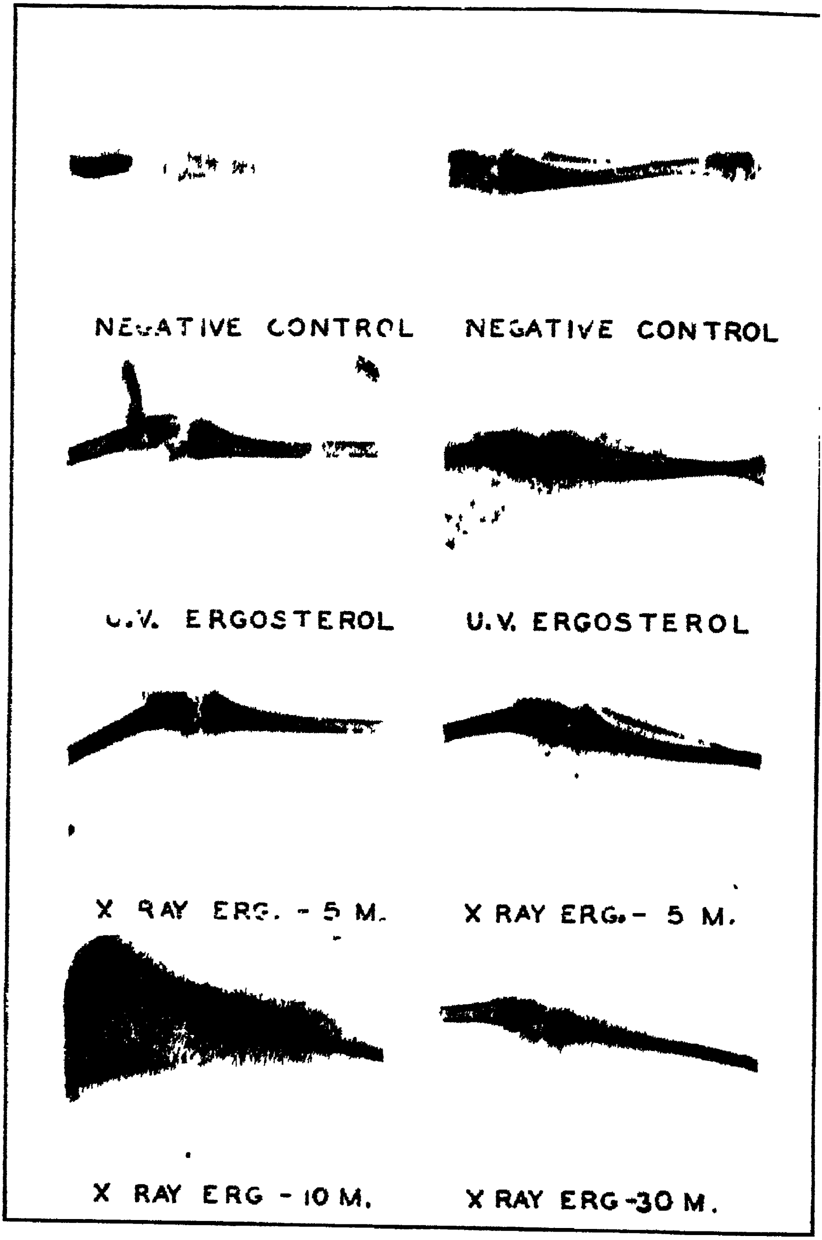


Fig. 5—Typical X-Ray Pictures of Rats Used in Feeding Experiments with Soft X-Ray Activated Ergosterol

TABLE II

Results of Animal Experiments

GROUP	NO. OF RATS	ADDITION TO DIET	AV. WT. TEST PERIOD		AVERAGE GAIN	GENERAL CONDITION
			BEGINNING	END		
			SERIES I			
I	6	0.002 mg. u. v. irradiated ergosterol	81.2	100.7	19.5	Healing evidenced by general condition, X-ray pictures, and dissections
II	6	0.002 mg ergosterol irradiated soft X-rays, 50,000 V., 10 M. A., 11.5 min.	79.0	89.7	10.7	Evidence of healing, but results not so regular and general condition of rats not as good as Group I
III	6	0.002 mg plain ergosterol	79.9	82.2	2.3	Condition poor
IV	4	No addition	80.8 (4 rats)	81.0 (3 rats)	1.0	One rat died, two lost weight, one gained 4 g
I	4	0.002 mg ergosterol activated with u. v. in Fleischmann Laboratories	62.0	81.0	19.0	General condition good Healing ++++.
II	1	0.002 mg ergosterol irradiated soft X-rays, 10,000 V., 10 M. A., 5 min	61.8	75.1	13.3	General condition good. Healing ++.
III	4	0.002 mg. ergosterol irradiated soft X-rays, 40,000 V. 10 M. A., 10 min	62.1	72.2	10.1	General condition fair Healing ++.
IV	1	0.002 mg. ergosterol irradiated soft X-rays, 10,000 V., 10 M. A., 30 min	62.2	65.5	3.2	General condition fair Healing +
VI	4	No addition	62.1	63.0	0.6	General condition poor. No healing

food containing the ergosterol was fed to each rat. Later the solution was poured over a very small amount of sugar (sugar was used simply to prevent the ergosterol's adhering to the dish), and a suspension in water was fed with a medicine dropper. This was the more satisfactory procedure.

After a test period of six days, X-ray pictures were taken. Feeding was continued for a second period of six days in order to secure more decisive pictures. At the end of the test period at least one rat from each group was examined post-mortem, and in some cases "line tests" were made to check the interpretation of the X-ray photographs. The results of the experiments, as recorded in Table II and illustrated in the photographs of Figure 5, indicate that the ergosterol exposed to soft X-rays gave antirachitic protection comparable to that obtained with ultra-violet activated ergosterol.

DISCUSSION OF RESULTS

The authors believe the results of their experiments indicate that X-rays in such a region as to be absorbed appreciably by ergosterol have appreciable activating effect.

Since less protection was obtained in the Series II feeding experiments in the case of the longer exposures to soft X-rays, this suggests that the prolonged exposures may have a de-activating effect comparable to that obtained in the ultra-violet region.

In regard to the work of Reinhard and Buchwald (3), in which they found an effect of hard X-rays on cholesterol especially noticeable when a chloroform solvent was used, the authors are inclined to agree with the explanation of Hieger (7) that the effect is probably one on the solvent. The X-ray absorption of chlorine in the hard X-ray region is much higher than that of any of the elements in cholesterol or ergosterol. In any solution in which there is present in the solvent a considerable proportion of an element of higher atomic weight than the carbon, hydrogen, and oxygen of cholesterol and ergosterol, increased absorption would be expected in the solvent as compared with the cholesterol or ergosterol. It is possible that the work of Hopf and Ludwig (9), who found apparent destruction of the antirachitic activity of foods by irradiation with X-rays, may be explained by the action of X-rays on elements in the food of higher atomic weight than carbon, hydrogen, and oxygen.

In considering the results of those who believe that they have obtained activation or chemical changes with hard X-rays, we must remember first that the present paper does not suggest that activation or chemical change does not take place in the hard X-ray region, but only that—because of decreased absorption—one would expect changes to take place in this region at an almost negligible rate; and second, that when the technique involves the use of glass containers (as in the work of Reinhard and Buchwald), one must take into consideration the fact that certain kinds of glass give brilliant ultra-violet fluorescence in the presence of X-rays. In our own work a number of different containers have been used, with consistent results.

The present paper does not attempt to prove, of course, that the action of the soft X-rays is a primary one. Whether the change is brought about by an unbalance of the molecule through the removal of inner electrons, by impacts of ejected photoelectrons,[1] or by secondary radiations, is a question still to be answered—exactly as the question of how ultra-violet light activates ergosterol is still an open one. The authors hope to continue experiments along these lines, and to carry out further experiments with monochromatic radiations, which will enable them to correlate the extent of activation with the absorption in different portions of the X-ray spectrum.

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AN IMPROVED SINGLE QUARTZ FIBER ELECTROSCOPE

By ROBERT B. WITHROW

SUMMARY

A vacuum-type quartz fiber electroscope is described with plates so arranged that the fiber cannot strike them. It is possible to obtain sensitivities up to 11,000 divisions per volt, or greater if greater magnifications are used, together with stability and ease of operation.

INTRODUCTION

Quartz fiber electroscopes of the type described by Professor Swann (1), utilizing free-hanging fibers between parallel plates, are troublesome because of the tendency of the fiber to strike one of the plates. If the fiber is over 5μ in diameter and is operated in air, it is possible to free it readily and continue work; but smaller fibers, operated in a vacuum, are usually destroyed in attempting to free them. The author has constructed two instruments of the type described by Dr. Swann, and has been unable to mount and use fibers smaller than 3μ in them. It is the purpose of this paper to describe an instrument having the sensitivity, stability, and linearity of response of the parallel-plate type, but using inclined plates which the fiber cannot strike.

EXPERIMENTAL

The instrument (Figure 1) consists of a brass base (12) into which is cut a groove (17) filled with wax (made of one part beeswax to four of resin), sealing the case (5) to the base. Into the top of the case (5) is sealed a quartz insulating tube (2) of 4 mm., inside diameter. The points marked (15) are sealed with hard DeKotinsky cement. Somewhat better seals may be made by wetting the quartz with a concentrated solution of platinic chloride, heating the quartz to red heat, copper plating over the platinum, and soldering between the copper and the case. The solder should be covered with a thin layer of hard wax to fill the pores and make the seal vacuum tight.

The windows (9) are sealed with wax into rectangular slots milled into the case. The window in the front of the case, through which the fiber is observed with a microscope, should be sunk in as far as possible, so that high-power objectives with small working distances may be used.

Another window, not shown in the diagram, was added later, just to the left of the front window. It is about one-half inch in diameter, and is used for illuminating the fiber for observation by reflected light. Any fiber of smaller diameter than about 2μ is below the resolving power of the 24 mm. microscope objective used by the author, and therefore appears by transmitted light as a gray haze or shadow instead of a sharply defined image. Observation by reflected light eliminates the difficulty, as in the ultra-microscope. It was not possible to illuminate the fiber through the window used for observation, because the light scattered by the window obscured that reflected by the fiber itself.

A bakelite block (19) is used for insulating the lead-in wires (soldered quartz tubes, as described above, could be used). The hole (18) is for evacuating the electroscope. The fiber is soldered to a pin in a small brass block (7), which is supported by a quartz insulator (13) and brass post (6). An enlarged view of the plates and fiber mounting is shown in Figure 2. It is apparent that the fiber (which is about $\frac{7}{8}$ inch long) cannot touch either plate, no matter how far it swings. The plates (10) are mounted a fixed distance apart on bakelite posts (11).

Figure 3 shows the complete assembly, including the microscope and illuminating apparatus, mounted upon a tripod, so that the electroscope may be leveled. The electroscope is mounted on the stage of the microscope, and may be moved back and forth across the stage by the screw (G) to adjust for zero.

The quartz fibers are produced by the drift method in a quiet oxyhydrogen flame about six inches long. When a good one has been selected, it is mounted between the greased ends of a U-shaped wire, and sputtered with platinum or gold in a 0.1 to 0.5 mm. vacuum (the lower pressure is preferred) at 1,000 volts. It requires about one minute to get a coating just thick

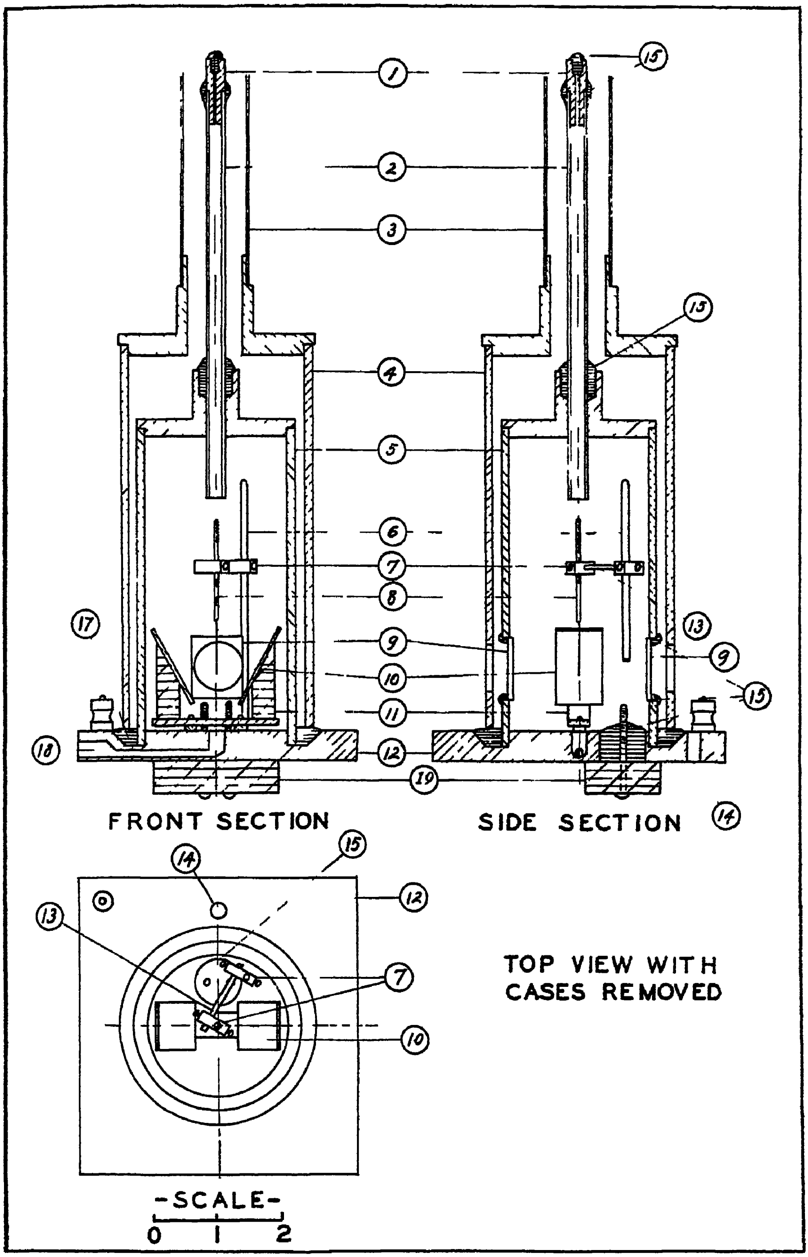

Fig 1 —Electroscope Assembly

enough to make the fibers conducting. Fibers more heavily coated are sluggish, and sometimes acquire a permanent set. After the fiber is sputtered, it is soldered with Wood's metal (while still mounted on the U-shaped wire) to its supporting pin. Heating is accomplished by radiation, without touching the fiber or pin, by using a small heating coil operated at dull

TABLE I

Calibration of Quartz Fiber Electroscope

	E	D_0	D_1	D		E	D_0	D_1	D
E_p 17	002	4	22	18	E 15 5	01	0	14	14
	004	8	48	40		02	0	29	29
	006	13	75	62		03	0	42	42
S 11,000	008	15	105	90	S 1320	04	0	56	56
	010					05	0	69	69
						06	0	83	83
						07	0	96	96
						08	0	109	109
						09	0	122	122
E_p 16 5	002	5	18	13	E_p 15 0	01	0	10	10
	004	5	30	25		02	0	20	20
	006	5	38	32		03	0	30	30
S 5,350	008	3	47	44	S 935	04	0	39	39
	010	2	55	53		05	0	48	48
	012	2	65	63		06	0	57	57
	014	2	77	75		07	0	67	67
	016	3	88	85		08	0	77	77
	018	5	100	95		09	0	85	85
	020	7	110	103		10	0	94	94
E_p 16 0	004	0	12	12					
	008	0	21	21					
	012	5	38	33					
S 2,560	016	4	47	43					
	020	2	57	55					
	024	0	65	65					
	028	0	75	75					
	032	0	85	85					
	036	0	95	95					
	040	0	105	105					

Notation:

E = Potential applied to fiber, in volts
D_0 = Zero reading.
D_1 = Reading with potential applied
D = Actual deflection of fiber in 004 mm. units
S = Sensitivity of instrument, divisions per volt.
E_p = Potential between plates, volts.

red temperature and held about one-fourth inch from the fiber. A dilute solution of zinc chloride is used as a flux. The soldered end of the fiber is then pulled away from the wire U and the brass pin is inserted in its socket on the quartz mounting rod in the electroscope. The assembly is lowered into position and the lower end of the fiber freed from the U wire with a pair of especially honed surgical scissors. Immediately after the fiber is cut, the inner case is carefully lowered into position, so that air currents will not carry the fiber up around the mounting pin.

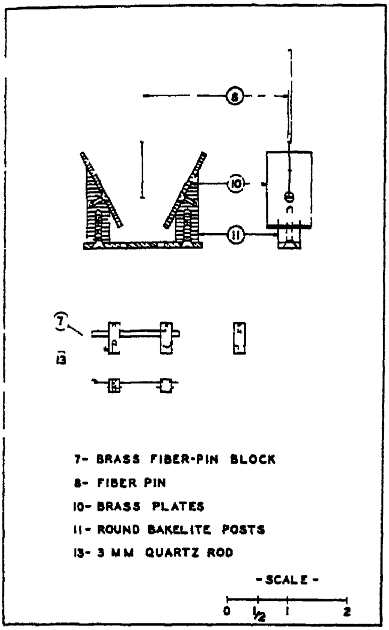

Fig. 2—Method of Mounting Plates

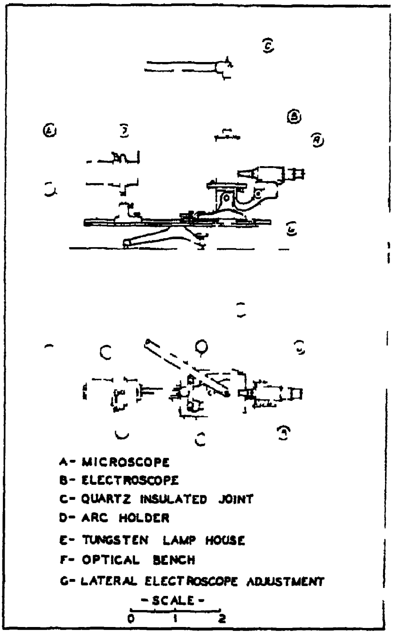

Fig. 3—Complete Equipment, Set Up For Operation

Table I gives data for the characteristic curves shown in Figure 4. From this table it is apparent that at a sensitivity of 5,350 divisions per volt and a potential of 16½ volts on the plates, the zero shift is only about five divisions maximum. Raising the potential on the plates to 17 volts caused the zero to shift badly, but this was probably due to maladjustment.

Since it was not necessary in the writer's work to use the instrument at this high sensitivity, great care was not exercised in its adjustment. By using a microscope of increased magnification, it would be possible to attain maximum sensitivities of 20,000 to 30,000 divisions per volt.

The period of the fiber decreases almost proportionally with decreases in pressure within the range of pressures used (it was not possible with the pump used to obtain pressures below 0.1 mm.). At the highest sensitivity, periods below 10 seconds were obtained. At a sensitivity of 3,000 divisions per volt, the instrument acts very much like a voltmeter. It is stable, its zero is constant, and it is not greatly affected by vibrations in the building. It is superior to quadrant electrometers in its short period, great stability, lack of zero drift, and small electrical capacity; and it is much easier to work with than the usual parallel-plate type of instrument.

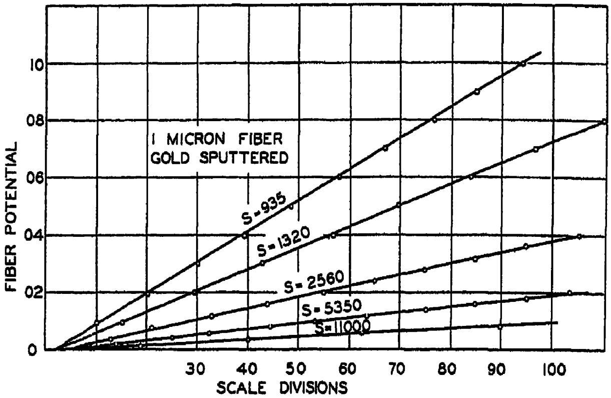

Fig. 4—Sensitivity Curves

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THE LETHAL ACTION OF SOFT X-RAYS ON MICROÖRGANISMS[1]

By GEORGE N. BURGER

SUMMARY

Soft x-rays in the region 2.0 to 0.5 Ångstrom units are found to have marked lethal action for staphylococcus aureus, saccharomyces cerivisiae, bacillus subtilis, bacillus coli, bacillus tuberculosis bovine type, and staphylococcus albus. At 30,000 volts, 100 per cent killing of Petri dish cultures is obtained upon 3 to 10 minutes exposure, at 3 to 4 cms. from the window of the tube.

The soft radiations, lethal for bacteria, are demonstrated to penetrate with considerable intensity through two to three millimeters of agar.

The lethal action increases with the tube voltage, but not in proportion to the total energy emitted by the tube.

Since the early work of Bucky on soft X-rays, a number of investigators have found differences in the biological action of soft and hard X-rays. Most of these investigators have been interested in the therapeutic effects of these radiations. A few have studied the action of radiations on microorganisms. Data in regard to the latter are quite incomplete at the present time, however. It is the purpose of this paper to present additional data regarding the lethal action of soft X-rays on various microorganisms. In addition to purely qualitative results, an attempt has been made to add quantitative data regarding the comparative effect of different wave-lengths.

REVIEW OF THE LITERATURE

The fact that soft X-rays (that is, X-rays lying near the border line between the ultra-violet and X-ray regions) produce somewhat different biological effects than ordinary X-rays was first emphasized by Bucky. Bucky's work is discussed by Kirsch (1). He conducted further experiments in which he

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[1] Assisted by a grant from the National Research Council, Committee on Radiations

found that three minute irradiation by wave-lengths from 1.2 to 2 Å. at 5 cm. from the tube window causes erythema. Russ (2), in 1923, studied the effect of different wave-lengths of X-rays upon animal tissues and found that there is a variation in the effect with wave-length.

Schepmann and Flecke (3) were the first to investigate the lethal action of soft X-rays on bacteria. They found that equal doses of hard X-rays produced only about one half the effect of soft X-rays on *staphylococcus aureus, bacillus coli, bacillus pyocyaneus, bacillus prodigiosus,* and *bacillus mesentericus.*

Following up his earlier work, Bucky published a paper (4) in which he stated that erythema could be produced with soft X-rays with one-eighth of the energy required in the hard X-ray region.

J. J. Trillat (5) determined in a qualitative way that B. prodigiosus is killed by the long-wave, or soft, radiations. Gaertner (6) also found, in a rough way, an increased lethal effect of soft X-rays on microörganisms as compared with hard rays, and stressed the importance of further investigations along this line.

About 1927, H. Fuhs (7) attempted the treatment of various skin diseases with soft X-rays, being led to this application by the knowledge of their lesser penetrating power and greater lethal action upon bacteria. With a water-cooled Müller tube, he claimed to have treated successfully tuberculosis verrucosa cutis, mycosis fungoides, benign skin tumors, etc.

In 1928 and 1929 Lacassagne (8 and 9), Holweck (10), and Mme. Curie (11) published data regarding the quantitative effect of soft X-rays upon certain microörganisms. With radiations in the region from 4 to 8 Å., they calculated the number of quanta of energy necessary to kill a single bacterium. They found that in the case of *bacillus pyocyaneus* a single quantum sufficed to kill a bacterium at 4 Å., while at least 4 quanta were necessary at 8 Å.

EXPERIMENTAL

EQUIPMENT

The equipment used in our experiments consisted of a Shearer tube, with interchangeable targets and an aluminum window, operated from a source of mechanically-rectified, high voltage direct current. Voltages from 5,000 upward were available.

The operating voltage was measured by means of a spark gap. The tube was highly evacuated, and the pressure adjusted until the tube current was from 5 to 10 m.a. at each voltage used.

During the course of the work it was found necessary to dismantle the tube from time to time in order to clean from the window the coating of metal sputtered from the target. If the sputtered coating was allowed to collect to an appreciable thickness, its absorption for the required long-wave radiations became so great that no biological effects could be obtained.

TECHNIQUE OF HANDLING MICROORGANISMS

In a majority of the experiments, broth cultures of the microörganisms were inoculated on sterile media in Petri dishes, and these dishes were exposed, open, under the window of the X-ray tube. The distance was so regulated that but a small portion of the dish was irradiated. Thus, the non-irradiated area of the same plate acted as a control.

RESULTS WITH VARIOUS MICROORGANISMS

Staphylococcus Aureus—Agar plates inoculated with three to four drops of twenty-four hour broth cultures of *staphylococcus aureus* showed 25 per cent killing in three minutes at 15,000 volts, 10 m.a., 4 cm. distance from the tube window; 100 per cent killing in five minutes at 30,000 volts; and 100 per cent killing in two minutes at 35,000 volts. The relation between the extent of killing and the time of exposure at different tube voltages is shown in the accompanying graphs (Figure 1). These results were obtained with a molybdenum target in the tube.

Saccharomyces Cerivisiae—This organism proved to be somewhat more resistant to radiations than most of the bacteria used. With a silver target in the tube, 100 per cent killing was obtained in ten minutes at 35,000 volts, 10 m.a. tube current, 3 cm. distance. Voltages lower than this failed to show an appreciable effect.

Bacillus subtilis—*Bacillus subtilis* showed 80 to 90 per cent killing in ten minutes at 35,000 volts, 10 m.a., 4 cm. distance, with a silver target in the tube. A curve of extent of killing plotted against time of irradiation at 35,000 volts is shown in Figure 2.

Bacillus Coli—*Bacillus coli* X-rayed at 35,000 volts, 10 m.a., 4 cm. distance, with a silver target, showed 70 per cent killing in two minutes, and 100 per cent in five minutes. No killing was noted for exposures shorter than two minutes. In order to demonstrate that the radiations responsible for the lethal action would not penetrate glass, several plates were exposed with the covers in place. They showed no killing whatever.

Bacillus Tuberculosis, Bovine Type—Considerable difficulty was experienced with this organism on account of the fact that during exposure the plates became contaminated with molds and other organisms which developed more rapidly than the bacillus. For that reason, accurate counts could not be made, but it was evident that appreciable killing had taken place—at least during the longer exposures at 35,000 volts.

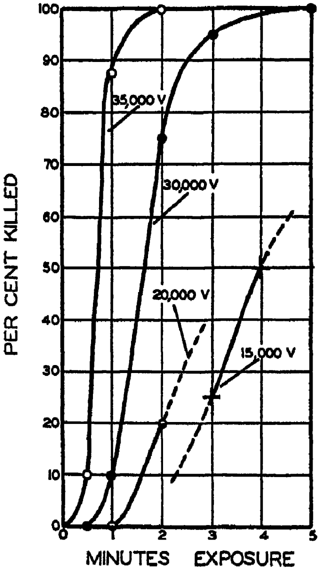

Fig. 1—Killing Curves, Staphylococcus Aureus

Staphylococcus Albus—The lethal action on this organism varied from 40 per cent killing in four minutes at 15,000 volts, 10 m.a., 4 cm. distance, to 100 per cent killing at 30,000 volts, four minutes, with the same tube current and distance. A silver target was used in these experiments. Curves of the lethal effect against voltage and time of exposure are shown in Figure 3.

EFFECT OF SOFTER (LONGER WAVE) RADIATIONS

In the experiments thus far described, no killing was noted at voltages less than 15,000, and in some instances no effect was noted at voltages less than 30,000 to 35,000. While the shortest wave length emitted by the tube is limited by the voltage, according to the relation:

$$\lambda = \frac{12.35}{V}$$

where:

λ = wave length in Å.
V = tube voltage in kilovolts

from which one might interpret the lack of killing at low voltages to be due to insufficiently short wave lengths, it was believed, rather that the failure to obtain killing was due to the lower intensity at the lower voltages (the intensity of the X-ray beam varies as approximately the square of the tube voltage).

In order to test this explanation of the lack of killing at the low voltages, glass slides coated with agar and inoculated with surface cultures of *staphylococcus albus* were irradiated at 5,000 volts, 10 m.a., at a distance of 0.5 cm. from the tube window. In ten minutes these slides showed approximately 5 per cent killing, and in twenty minutes, considerably greater killing. In another experiment, Petri dishes exposed at 7,500 volts, 1 cm. distance from the tube window showed 95 per cent killing in twenty minutes. The assumption that lack of sufficient intensity caused previous failures to obtain killing at the lower tube voltages seemed justified.

EFFECT OF SOFT RADIATIONS ON THE NUTRIENT MEDIUM

In order to show that the lethal action on various microörganisms was actually a direct action on the microorganisms themselves rather than one on the medium irradiated, Petri dishes containing nutrient agar were exposed for ten minutes at 35,000 volts, 10 m.a., 3 cm. from the tube window and inoculated later with suspensions of *staphylococcus albus.* Similar Petri dishes, not irradiated, were inoculated and kept as controls. Since but a small area of the irradiated plates was actually exposed to X-rays, the plates themselves acted as their own controls, separate controls being kept merely as an additional check because of the remote possibilities that the effect of the irradiation might spread throughout the medium or that scattered rays might induce changes in parts of the medium outside the path of the X-ray beam. In no case could any consistent difference be noted in the growth of organisms on the irradiated and non-irradiated media.

As a further proof that the action is a direct one on the

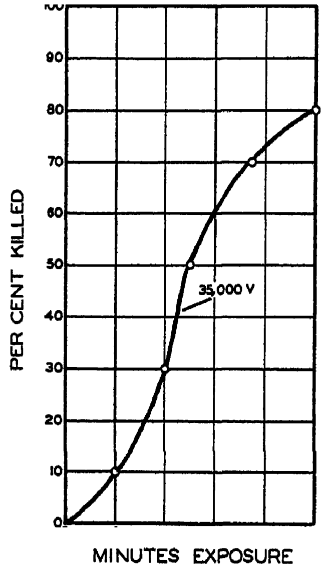

Fig. 2—Killing Curves, Bacillus Subtilis

organisms themselves, glass slides containing suspensions of *staphylococcus albus* were exposed to soft X-rays at various voltages and for various lengths of time. The lethal action varied from no killing at 25,000 volts, 10 m.a., 2½ cm. distance in one minute, to 100 per cent killing under the same conditions in five minutes, and practically 100 per cent killing under the same conditions at 40,000 volts in two minutes. This demonstrated that it is not necessary that the bacteria be exposed in the presence of a nutrient medium in order for the lethal action to take place.

PENETRATION OF THE LETHAL RADIATIONS

From the standpoint of the treatment of surface infections in skin diseases, the penetration of the lethal soft X-rays through human tissue is important. Reliable data regarding absorption of various types of tissue for X-rays at different wave-lengths are not available. Fortunately, however, nutrient agar, having somewhat the same constituent elements (carbon, hydrogen, oxygen, nitrogen, etc.) as skin and muscle tissue, should have somewhat similar X-ray absorption (it being well established that X-ray absorption is dependent almost entirely on the elements present in chemical compounds, and practically independent of their state of chemical combination). It is possible, therefore, to determine roughly the penetration of the lethal radiations through human tissue by finding experimentally the penetration of these radiations through nutrient agar.

In one series of experiments, broth suspensions of *staphylococcus albus* were inoculated into tubes of agar, each of which contained about 6 cc. of agar. The medium was then poured into petri dishes, the bacteria being distributed throughout the body of the medium by this method. The amount of medium used was sufficient to fill an ordinary Petri dish to a depth of about 1 mm. It was found that if one tube was used per dish, 100 per cent killing could be obtained at 35,000 volts, 10 m.a., 4 cm. tube distance in about five minutes. With two or three tubes, the extent of killing was reduced to about 90 per cent in the same time. Thus, a sufficient percentage of the lethal radiant energy remains at a depth of 3 mm. to cause appreciable killing of microörganisms.

In a second series of experiments, Petri dishes containing thin layers of nutrient medium inoculated on their surface with broth cultures of *staphylococcus albus* were covered with layers of agar of varying thicknesses. Exposures were made under the same conditions as in the preceding series of experiments. One hundred per cent killing was obtained through 2 mm. of agar, and about 95 per cent killing through 3 mm. of agar. These results are consistent with those of the preceding experiments.

EPILATION EXPERIMENTS

The reactions to soft X-rays are delayed, as in the case of hard X-rays, for some two to three weeks after exposure. Long exposures are followed by marked epilation and sloughing of surface tissue. More moderate exposures are followed by erythema, with subsequent pigmentation. Very short exposures produced no superficial action whatever.

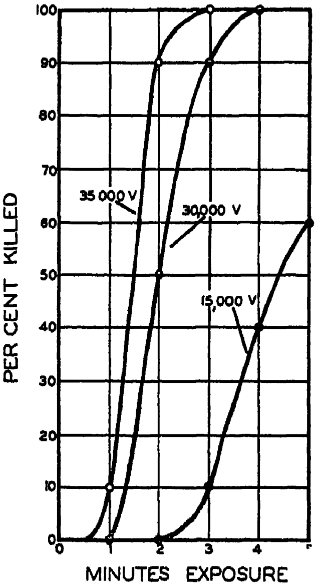

Fig. 3—Killing Curves, Staphylococcus Albus

In order to determine the required dosage for erythema and epilation, three series of mice were X-rayed on the abdomen at various voltages and for different lengths of time. A uniform time of about three weeks was required for the reaction to develop in the case of all animals surviving. Exposure of two to four minutes at 35,000 volts, 4 cm. distance produced noticeable epilation. Exposure of six to eight minutes resulted in marked reactions, with considerable sloughing of tissue; and exposures of fifteen minutes or more under the same conditions resulted consistently in death of the animals. Animals examined post mortem showed no evidence of injury to internal organs.

A similar experiment on rabbits resulted in epilation after exposures of ten minutes or more. On humans, erythema developed uniformly at 35,000 volts upon exposures of thirty seconds or more. Exposures of thirty seconds resulted in a comparatively slight reaction, while two to four minutes produced a rather severe reaction.

CONCLUSIONS

From the foregoing experiments it is apparent that soft X-rays have a much more marked lethal action upon various types of organisms than X-rays in the hard region.

The indications are that the softer the radiations, the greater is the lethal effect. In our experiments, the more pronounced action was found at higher tube voltages; but this may be explained by the greater total energy of emission at the higher voltages, especially inasmuch as molybdenum and silver targets were used in the tube, and a sheet of aluminum foil, acting as a filter, was used for the tube window.

The fact that the total energy of emission increases as approximately the square of the tube voltage, whereas the lethal action in these experiments increased at a much lower rate, seems to indicate that the softer (or longer wave) radiations actually have a much more pronounced bactericidal effect.

This is what one would expect from a consideration of X-ray absorption. Microörganisms are made up largely of carbon, hydrogen, nitrogen, and oxygen. Radiations included in the soft and hard X-ray regions all lie within the K absorption region of these elements. The total absorption of the microörganisms would, therefore, be expected to increase as approximately the cube of the wave-length, the relation for the total absorption of each of the constituent elements with regard to wave-length being:

$$\frac{\mu}{\rho} = \frac{CN^4 \lambda^z}{A} + \frac{\sigma}{\rho}$$

in which μ = linear absorption coefficient (absorption per unit thickness).
ρ = density of the element.
C = a constant, changing abruptly at certain wave-lengths in the spectrum, known as "absorption limits."
N = atomic number of the element.
λ = wave-length of X-rays used.
A = absolute mass of the absorbing atom.
σ = a constant representing the scattering of X-rays by the material
z = an exponent varying according to the wave-length and material from 2 to 3.

Since radiations cannot bring about effects unless they are absorbed by the material upon which they are acting, one would expect the lethal action to be proportional to the absorption, and therefore to be greater for long-wave (or soft) radiations than for short-wave (or hard) radiations.

Experiments on bacteria indicate that the lethal radiations are of sufficient hardness to penetrate through several millimeters of human tissue. This is advantageous from the therapeutic standpoint, since it should make possible their use for the treatment of superficial infections too deep-seated to be affected by the less penetrating ultra-violet radiations.

The experiments show that, with the apparatus used in this laboratory, the safe exposure for humans is about one half to two minutes at 35,000 volts, 10 m.a. Because of the large variation in equipment, however, this cannot be used as a standard of exposure for other types of apparatus, but is merely an indication of safe practice with the particular tube used and under the particular conditions of our experiments.

Note (Appended May, 1931): After this paper had been submitted, a paper by Ralph W. G. Wyckoff appeared in *Journ Exptl. Med.*, 52 (1930): 435 in which soft X-radiations from a tube with a tungsten target and a Lindemann window operated at 12 k.v. were found to kill *B. coli* and *B. aertrycke* in a linearly exponential fashion. Deductions from quantum considerations lead to the conclusion that when death resulted it was caused by the absorption of a single quantum. Since but about one twentieth as many cells were killed as quanta were absorbed, it was deduced that the vital cell constituents involved in death occupy a volume less than 0.06 that of the cell.

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CELLOPHANE AND GELATINE FILTERS FOR THE ULTRA-VIOLET

By ROBERT B. WITHROW

SUMMARY

Dyed cellophane and gelatine filters for the region of the ultra-violet spectrum from 2,800 to 3,400 Ångstroms are described. These filters have sharp absorption limits, combined with stability. They can be prepared readily, and are inexpensive.

INTRODUCTION

When ultra-violet filters are used for investigating the biological effects of various regions of the ultra-violet, the correct interpretation of results depends upon the use of filters having sharp absorption limits. This is especially true of botanical investigations because of the exceedingly great sensitivity of plant tissues to the destructive action of the region beyond the limit of the sun's spectrum, that is, beyond wave-length 2,900 Ångströms. The irradiation of plants by artificial sources through filters which transmit some of this lethal region yields results which are easily misinterpreted.

The ultra-violet transmitting glasses now available for use in this region all transmit very appreciable amounts of energy in the region beyond 2,900 Ångströms. They have the added disadvantage that the point at which they absorb is beyond the control of the investigator except by choice of other glasses.

When small, intense sources of radiations are used, such as small quartz mercury arcs, sparks, carbon arcs, etc., it is possible to use quartz absorption cells filled with solutions having the desired characteristics. This type of filter lends itself to wide variation and control, and with proper choice of solutions has sharp absorption limits, but is not practicable for use with extended sources because of the cost of large filter cells. In biological experiments, there is a need for filters with sharp absorption limits which can be used with extended sources.

EXPERIMENTAL

The present paper describes a series of impregnated cellophane filters developed in this laboratory for investigating the effect of ultra-violet radiations on plants. They are easy to make, cheap, and have the advantages of special glasses for use with large sources, together with the ready control and possibility of variation of the liquid filters. These filters have been in use in plant investigations for over a year, and have proved highly satisfactory.

While the filters described here cover only the small range of the ultra-violet at the limit of the sun's spectrum, frequently called the "stimulating region," the method is applicable to any region from 2,200 Ångstroms to at least 8,000 Ångstróms, and possibly much further.

Saunders (1) describes a liquid filter of a water solution of one-fifth molar potassium hydrogen phthalate, which has a very sharp absorption band, absorbing practically all radiations of wave-length shorter than about 3,050 Ångstroms. Using cellophane, which is quite transparent to the ultra-violet spectrum in this general region, it was found that a filter could be prepared by soaking it in such a solution. This new cellophane filter had much the same absorption characteristics as the solution filter, but in the more convenient form of a solid sheet.

The success obtained with potassium hydrogen phthalate led to the investigation of many other compounds in the hope of finding other filters having sharp absorption limits at different points in the region from 2,900 to 3,200 Ångstroms. The following compounds were found to have undesirable characteristics: salicylic acid, phenylacetic acid, hydrocinnamic acid, malonic acid, m-nitrobenzoic acid, anthranilic acid, tropic acid, picric acid, urea, phenol, naphthol, para-nitroso-dimethyl aniline, phenanthrene, and trinitrobenzene.

The potassium and sodium salts of many of the organic acids were studied, as well as their alcoholic solutions. Cellophane, however, does not seem to absorb substances from alcoholic solutions sufficiently to give good filtering characteristics. Some compounds, as potassium salicylate, form good filters in this region but rapidly deteriorate under the action of ultra-violet irradiation.

Three practicable filters were obtained by soaking cellophane in the solutions listed below.

No. 1.	Sodium benzoate	80 g.
	Water	1 l.
	Glycerin	125 c.c.
No. 2.	Potassium acid phthalate	40 g.
	Water	1 l.
	Glycerin	125 c.c.
No. 3.	Cinnamic acid	30 g.
	Potassium hydroxide	10 g.
	Water	1 l.
	Glycerin	125 c.c.

No. 600 DuPont cellophane is cut to the desired size and allowed to soak in the solution for at least twelve hours. Glycerin is added to the solutions to keep the cellophane pliable, since without the glycerin it becomes brittle and cracks. Technical glycerin is sufficiently transparent in the ultra-violet region.

In making up solutions for filter No. 3, an excess of acid is added and the solution filtered. In every case, the sodium or potassium salts may be used interchangeably without appreciable alteration in the absorption limit. The preparation of the filters is not critical since the absorption limit in each case is very sharp and not greatly affected by small variations in the concentration of the solution. If the solutions for filters No. 1 and No. 2 are too concentrated, the salts precipitate on the surface of the sheet and form white spots. It has been found that the thickness of the cellophane is not as important a factor as concentration, which indicates that the dissolved substances are carried on the surface of the cellophane rather than in the material itself. After the sheets of cellophane have soaked for the required time, they are hung up to dry, and after drying are ready for use.

Gelatine is about as transparent as cellophane in the ultraviolet, and may also be used as a solid medium for holding the solution. Pure, powdered gelatine is dissolved in the above filter solutions and the liquid spread out over sheets of quartz,

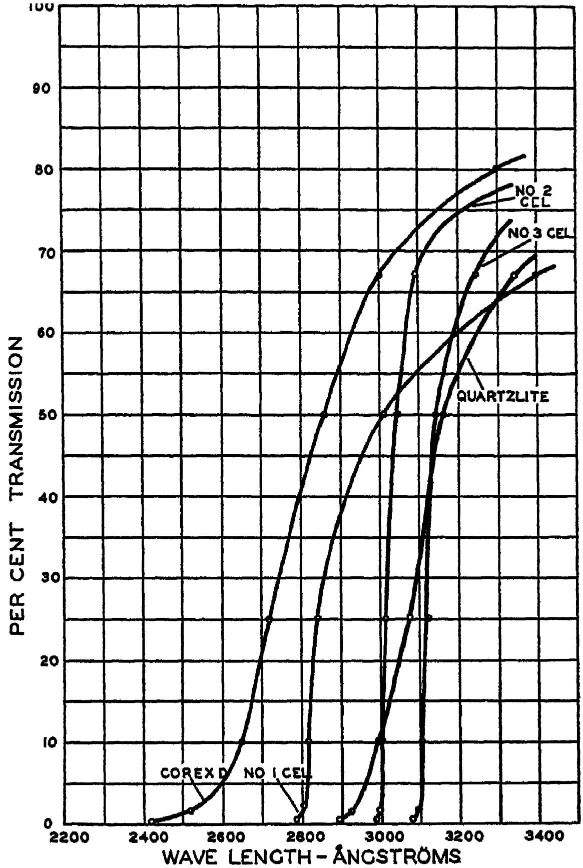

Fig. 1—Transmission Characteristics of Various Filters.

Corex D, Uviol, etc., and allowed to dry as in the making of photographic plates.

The resulting filter is superior to the cellophane type in that it is mechanically stronger and the surface is smoother. When the gelatine filter has deteriorated under prolonged irradiation, it may be removed with hot water and a new layer flowed on from a stock solution. Instead of using several sheets of material for increased filtering, as with cellophane, it is merely necessary to flow several layers onto the glass and thereby increase the thickness of the film.

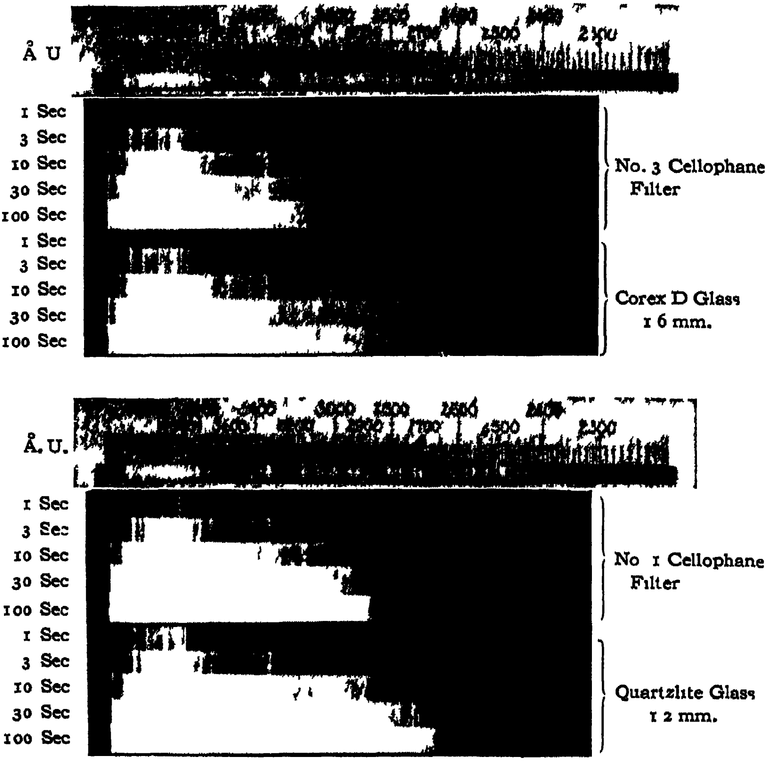

Fig. 2.—Comparison of the Sharpness of Absorption Limits of Impregnated Cellophane Filters and Glass Filters.

It has been found that phthalic acid is too insoluble to be sufficiently absorbed by cellophane, but that by using it as a saturated solution with gelatine, it is possible to obtain a filter with a transmission limit between those of No. 1 and No. 2.

In all cases observed, prolonged ultra-violet irradiation increased the absorption and shifted the absorption limit toward the visible. Cellophane acquires a light tan color upon prolonged irradiation which increases the general absorption of the ultra-violet. Thus, deterioration of the filters is characterized by decreased transmission and a shift of the absorption limit toward longer wave lengths.

Because of this slow deterioration under intense irradiation, it is advisable to use a primary filter of glass, such as Corex D, which is comparatively free from deterioration. This absorbs most of the energy in the short-wave ultra-violet region. The cellophane or gelatine filter then sharpens the absorption limit, practically eliminating transmission beyond the limit of the particular filter used.

In this laboratory, the cellophane type of filter base has been used mainly with the Cooper-Hewitt 50-inch low pressure type mercury arc, made with Corex D or Uviol glass. The lamp tube itself acts as an initial filter. Two sheets of No. 1 filter are required to absorb practically all radiations of wave length shorter than the 2,890 Ångstrom mercury line, two sheets of No. 2 to remove practically all beyond the 3,130 Ångstrom line, and three sheets of No. 3 to remove practically all beyond the 3,340 Ångstrom line. When a quartz mercury arc or impregnated carbon arc is used as a source with a plate of Corex D as the primary filter, the same number of layers of cellophane filters is required.

The curves in Figure 1 give the per cent transmission of the three cellophane filters for single thicknesses, and of 1.6 mm. Corex D and 1.2 mm. Quartzlite glasses. The cellophane filters have steeper curves and do not round off in the region of low transmission.

Figure 2 is a series of spectrograms comparing two of the cellophane filters with two ultra-violet transmitting glasses which have been much used by various workers. All these spectrograms were taken under the same conditions, with a tungsten spark as a source. The exposures were 1, 3, 10, 30, and 100 seconds.

By using compounds other than those mentioned, it should be possible to develop filters of this type for all regions of the visible and untra-violet.

The references below will be found useful in the development of filters of the type described in this paper.

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THE DESTRUCTION OF VITAMIN A BY ULTRA-VIOLET RADIATIONS

By ROBERT J. NORRIS

SUMMARY

1. Biological and colorimetric assays of oils for vitamin A do not appear to agree throughout the course of inactivation by irradiation with ultra-violet light. Oils irradiated until biologically inactive still showed 72 to 75 per cent of their original vitamin potency as judged colorimetrically.

2. Destruction of biological activity by irradiation was found to proceed rapidly after an initial induction period, in which there was very slight or no inactivation. Destruction of the chromogenic substance began immediately upon irradiation, without an induction period.

3. The author concludes that the chromogenic substance is not vitamin A itself, but some substance closely associated with it.

REVIEW OF THE LITERATURE

With the general recognition of the importance of vitamin D in food products, full ultra-violet or selective irradiation methods have been introduced to increase greatly the content of vitamin D in foods. Usually these food products contain more or less vitamin A, and the effect upon this latter factor must be taken into consideration.

Zilva (1) early pointed out the destructive action of ultra-violet rays upon the fat-soluble factor in butter. Holmes and Pigott (2) state that ordinary diffuse daylight passing through flint glass bottles is capable of destroying vitamin A. Peacock (3) found similar results, and noted a change in the absorption spectra of irradiated oils. Morton and Heilbron (4) had pointed out that absorption at 3280 Å. in cod-liver oils was characteristic of vitamin A, and that this absorption band disappeared when the vitamin was destroyed.

Titus, Hughes, Henshaw, and Fitch (5) found irradiated milk lost to a great extent its vitamin A content. Many other investigators have called attention to the deleterious action of light upon vitamin A.

On the other hand, Nabarro and Hickman (6) found that for such short periods of irradiation as thirty seconds with full ultra-violet there was little if any destruction of vitamin A. Supplee and Dow (7) concluded that there was little loss of vitamin A during periods of irradiation sufficient to markedly increase the content of vitamin D.

It would seem from these various results that the intensity and spectral distribution of the light, as well as the time of irradiation, is worthy of more critical attention.

In our preliminary work we were most interested in the stability of the vitamin to various radiations. It was soon noticed that vitamin A, at least in cod-liver oil, was relatively stable to ultra-violet rays.

It had been planned to investigate the stability of the vitamin under various conditions of irradiation in a number of different food products. It was found, however, that there is a wide discrepancy between the biological and colorimetric methods for determining the extent of destruction of vitamin A, and it was therefore decided to undertake first a series of biological and colorimetric tests on ultra-violet irradiated cod-liver oil.

An examination of the literature showed no critical study of the correlation between biological and colorimetric tests of irradiated oils. Evers (8) investigated the effect of light, and particularly the ultra-violet rays, upon vitamin A in cod-liver oil. He found little or no change in activity up to 2 hours and a slow change thereafter, but he did not determine the vitamin content by biological assay. His result contrasts strongly with those of Peacock (3), who found complete destruction in as little as ten hours with sunlight passing through two layers of glass.

Willimott and Wokes (9) also studied the action of ultra-violet upon vitamin A and found rapid destruction, which they followed by the color reaction. They irradiated their oil in open vessels at 3 in. from the lamp, the temperature of the oil rising to 70° C. (17). It would seem hardly fair under these conditions to ascribe the entire action to the radiations. The combination of relatively high temperature, ozone, and oxygen might play as much or greater role than the light itself in destroying the vitamin or the chromogenic substance. They state that animal experiments "are being carried out" to determine whether or not the biological potency reaches zero at the same time that the color test shows 100 per cent destruction of the vitamin, but the results of these tests are not given. In view of the following experiments, we believe they missed important facts that could have been noted had they followed the progress of destruction by parallel biological and colorimetric assays.

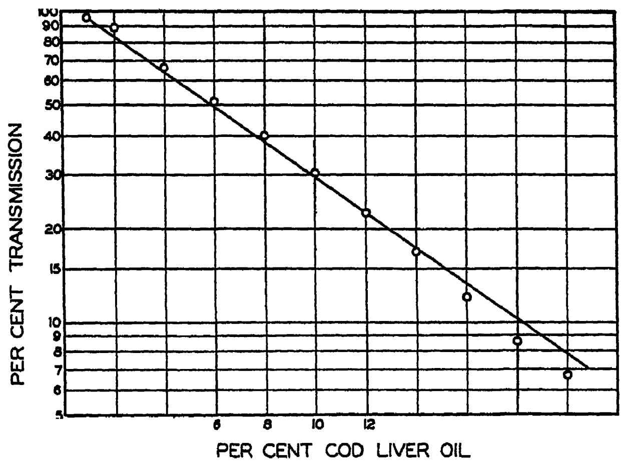

Fig. 1—Dilution Curve of Cod Liver Oil in Chloroform as Determined by the Density of the Blue Color at 6000-6170 Å Developed with Antimony Trichloride.

EXPERIMENTAL

DETAILS OF IRRADIATION

The irradiation of the oil was carried out as follows: 50 c.c. of cod-liver oil was placed inside a closed quartz cylinder 7 cm. in diameter. The air was completely expelled by a current of carbon dioxide, and the chamber sealed off. The quartz cylinder was mounted upon a revolving shaft, 20 cm. below the ultraviolet source, and driven at 30 to 45 R.P.M., which thoroughly agitated the oil and carried around a very thin film of oil on the inner surface of the cylinder directly in the path of the rays. A 500 watt, Hanovia, air-cooled quartz mercury arc was used as a source. The cylinder was cooled by a blast of air from a fan located beneath it, the temperature rise never being greater than 15° C.

Various times of exposure ranging from one-half to thirty-two hours were used, the oil being withdrawn and preserved in the dark at about 4° C. The activity of these samples was determined by both colorimetric and biological assay, the colorimetric determinations being made at the close of the feeding experiments.

BIOLOGICAL ASSAY

Rats of 35-45 grams initial weight were used. They were placed upon a vitamin A-free diet of the following composition:

Casein (Inactivated)	20%
Wheat Starch	70%
Dried Yeast	5%
Salt Mixture (McCollum's 185)	4%
Sodium Chloride	1%
Irradiated Ergosterol	.001%

After about three weeks, the animals showed typical symptoms of vitamin A deficiency, and when no further gain in weight after five days had occurred, feeding of the irradiated oil was begun. Preliminary experiments had shown the proper dosage levels corresponding to various times of irradiation.

The entire experiment, exclusive of the preliminary work, included two hundred and sixty animals, divided into fifty-two groups of five animals each. Each group received varying dosages of the irradiated oil. The doses administered ranged both below and above the normal requirement, and in this way a definite minimum dosage could be determined for each irradiation period.

The irradiated oil was made up in varying concentrations in inactivated olive oil and a constant quantity (1 c.c.) fed daily to each animal. After a short time the animals ate all of the diet in a satisfactory manner, until a few days before death.

The feeding was continued for five weeks after the initial symptoms of vitamin deficiency had become apparent. At the end of this time the average weekly gain over the entire period was determined for each dosage level.

In the case of insufficient dosage, many of the animals died far short of the five week period, and these results are averaged at the time of death. This always gave a negative quantity and is of no significance for comparison except to show insufficient vitamin.

The analysis of the growth curves is particularly difficult. Many investigators have shown that there is no correlation between the rate of growth and dosage, except at minimal values. Instead of attempting to judge vitamin content on the basis of equal acceleration of growth, we have taken the minimum quantity which would sustain life and produce a slow rate of growth. Even with this method the results are not as accurate as might be desired, and it would have necessitated an extremely large number of animals, with dosages very close together, to have given greater consistency. The results of the biological assay are compiled in Table I.

COLORIMETRIC ASSAY

In the colorimetric assay of these oils we used the technique of Carr and Price, as described by Cocking and Price (10). Instead of the usual Lovibond tintometer, a Bausch and Lomb Martens' polarizing spectrophotometer was used.

It was found impossible to employ the usual cells supplied with this instrument, and new ones were constructed, one cm. in thickness, the total volume of the cell being 3.5 c.c. Three tenths c.c. of the chloroform oil solution was placed directly in the cell and 3 c.c. of the antimony trichloride reagent added quickly. The mixing was complete without the necessity of stirring, and the whole process from the time of adding the reagent to the completion of the reading could easily be carried out in less than thirty seconds.

The blue color produced in the antimony trichloride reaction with cod-liver oils gives a band the peak of which is located at 6170 Å. The instrument was adjusted to include the region from 6000-6170 Å. and the transmission value determined for a number of concentrations of cod-liver oil in chloroform solution. A dilution curve was thereby obtained, which when plotted on a logarithmic scale gave a straight line throughout the range of concentrations used.

E. A. Norris and Church (11) have called attention to the widely differing types of dilution curves obtained with different oils. We have found considerable difference between different oils but not to such a marked extent as reported by those investigators. This work will be presented in a subsequent paper. The present investigation has all been carried out with the same oil.

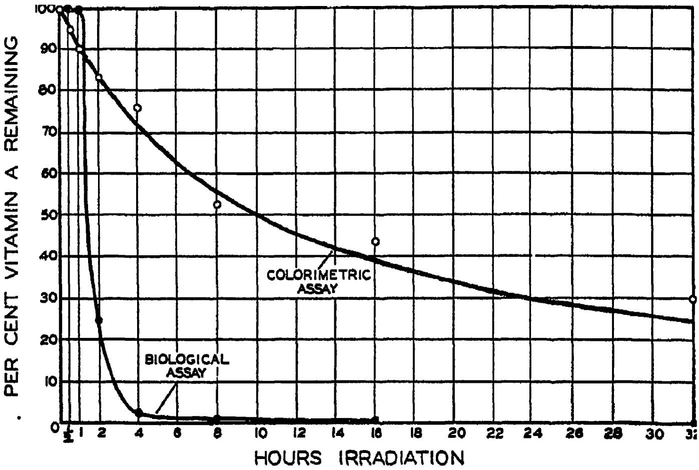

Fig. 2—Vitamin A Destruction By Ultra-Violet Irradiation

After a dilution curve had been established for this oil, it was merely necessary to compare the color test transmission of the treated oil with that of the untreated to determine the per cent of the chromogenic substance present. Figure 1 shows the type of dilution curve obtained with the standard brand of cod-liver oil used in this work. The transmission values, at a fixed concentration, for the oil irradiated for different time intervals were determined, and from this a curve was established showing the destruction as a function of time of irradiation (Figures 2 and 3).

In our preliminary work we were able to demonstrate an induction period up to two hours of irradiation, by both the colorimetric and biologic methods of assay. In the later work we were unable to confirm this refractory period by colorimetric determinations, but the biologic assays showed it strikingly. It is planned to investigate this matter more thoroughly in the near future.

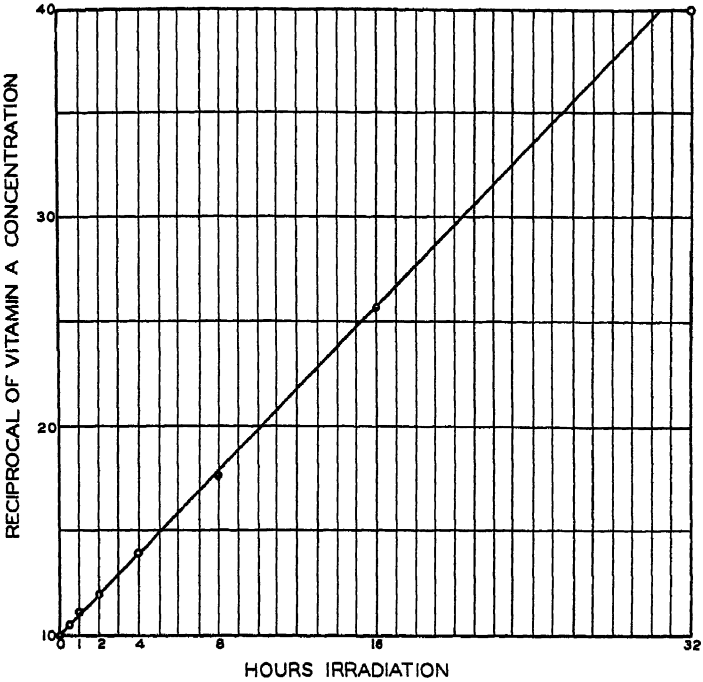

Fig. 3—Diminution of Vitamin A Concentration With Time of Irradiation as Determined by Colorimetric Assay. The points plotted here were obtained directly from the curve of figure 2.

When the reciprocal of the concentration of the chromogenic substance after different times of irradiation is plotted against

TABLE 1

Biologic Assay of Irradiated Oils

Group	Details of Oil		Average Weekly Gain of Group (Gms.)	Per Cent Survivors	No. Weeks Fed	Minimum Dosage	Per Cent of Vitamin Remaining
	Time of Irradiation	Dosage (mg.)					
1	0	0.5	− 4	20	5	....	....
2	0	1	0	80	5	....	
3	0	2	+10	80	5	....	....
4	0	4	+33	100	5	2 0	100
5	½ hr.	0.5	−10	20	5		
6	½ hr.	1.0	+ 9	20	5		
7	½ hr.	2.0	+0.6	40	5	....	....
8	½ hr.	4.0	+29	80	5		....
9	½ hr.	8.0	+39	100	5	....	....
10	½ hr.	16.0	+56	100	5	2 0	100
11	1 hr.	0.5	−17	40	5	....	....
12	1 hr.	1.0	−10	20	5	....	....
13	1 hr.	2.0	+0.2	80	5	....	....
14	1 hr.	4.0	+26	100	5	....	....
15	1 hr.	8.0	+26	100	5	....	....
16	1 hr.	16.0	+36	100	5	....	....
17	1 hr.	32.0	+69	100	5	2.0	100

18	2 hrs.	0.5	−21	60	5	....	....
19	2 hrs.	1.0	− 6	0	5	....	....
20	2 hrs.	2.0	+ 5	20	5	....	....
21	2 hrs.	4.0	−15	16	5	....	....
22	2 hrs.	8.0	+17	80	5	....	....
23	2 hrs.	16.0	+18	80	5	....	....
24	2 hrs.	32.0	+20	50	5	....	....
25	2 hrs.	64.0	+ 8	40	5	8.0	25.
26	4 hrs.	0.5	− 8	0	5	....	....
27	4 hrs.	1.0	−18	20	5	....	....
28	4 hrs.	2.0	−19	0	5	....	....
29	4 hrs.	4.0	−20	0	5	....	....
30	4 hrs.	8.0	−26	0	5	...	. ..
31	4 hrs.	16.0	−15	0	5	....	....
32	4 hrs.	32.0	−17	40	5	....	. .
33	4 hrs.	64 0	−14	16	5	..	..
34	4 hrs.	128.0	+46	100	5	128	1.5
35	8 hrs.	16.0	−17	20	5	....	....
36	8 hrs.	32.0	−15	20	5	...	....
37	8 hrs.	64.0	−19	60	5	....	....
38	8 hrs.	128.0	+ 4	80	5	128	1.5

TABLE I—Continued

Biologic Assay of Irradiated Oils

GROUP	DETAILS OF OIL		AVERAGE WEEKLY GAIN OF GROUP (Gms.)	PER CENT SURVIVORS	NO. WEEKS FED	MINIMUM DOSAGE	PER CENT OF VITAMIN REMAINING
	TIME OF IRRADIATION	DOSAGE (mg.)					
39	16 hrs.	8 0	−22	20	5	... .	....
40	16 hrs.	16.0	−24	0	5	... .	... .
41	16 hrs.	32.0	−22	20	5	..	....
42	16 hrs.	64 0	−19	0	5	. ..	....
43	16 hrs.	128.0	+ 3	60	5	. .	. .
44	16 hrs.	256 0	+63	100	5	128	1 5
45	32 hrs.	8 0	−17	0	5	....	....
46	32 hrs.	16 0	−16	0	5		.
47	32 hrs.	32 0	−18	0	5	... .	...
48	32 hrs.	64 0	−18	0	5	....	.
49	32 hrs.	128 0	−16	0	5	.	
50	32 hrs.	256 0	−17	0	5	256+	0 75

the time of exposure to the radiations, a straight line is obtained, indicating that the destruction follows a bimolecular reaction (Figure 3). Further experiments would be necessary in order to establish the nature of the reaction.

Our biological results are not sufficiently accurate for comparison on the same basis. Figure 2 shows the results of both methods of assay on the same irradiated oil. The initial induction period is well shown, and likewise the striking drop in vitamin content within the next hour, reaching practically zero after four hours of exposure. The colorimetric method still shows between 72-75 per cent of vitamin (if this can be accepted as a measure of vitamin activity at all) present after this time of irradiation.

Steudel and Peiser (12), Jones, Briod, Arzoomanian, and Christiansen (13), Bailey, Cannon, and Fisher (14) have failed to find complete agreement between the colorimetric and biologic methods of vitamin A assay. Hawk (15) found that exposure of cod-liver oil to radiations and air actually increased the blue color with the antimony trichloride reagent. Drummond later attempted to confirm this but was unable to do so. Norris and Church (16) have lately confirmed this point, but offer a different explanation of the phenomenon.

CONCLUSIONS

The results of these experiments lead one to the following conclusions:

1. Biologic and colorimetric methods of assay of irradiated cod liver oil show very wide discrepancy in the estimated vitamin A content.
2. Irradiated oils practically inactive in feeding experiments may show 50-75 per cent of the original vitamin activity as judged by the color reaction.
3. By biological tests a definite induction period of up to one hour has been found, during which practically no vitamin is destroyed by ultra-violet irradiation, followed by very rapid destruction in a subsequent equal interval of time.
4. Under the conditions of these experiments, practically

complete inactivation occurs after eight hours full ultra-violet irradiation, as judged by feeding experiments.

5. The destruction of the chromogenic substance has been shown to follow a bimolecular reaction, and no induction period has been demonstrated for it (except in one less accurate preliminary experiment).

6. The author believes that these results indicate that the chromogenic substance, while closely following vitamin "A", is not the vitamin itself, and hence that colorimetric measurement of this factor can not be relied upon under all conditions.

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VITAMIN D—A REVIEW

PART 1—ULTRA-VIOLET ABSORPTION SPECTRA IN RELATION TO VITAMIN D. SECTIONS A AND B

By JOHN R. LOOFBOUROW

SUMMARY OF PART I, SECTIONS A AND B

A. Early Attempts To Correlate Absorption Spectra and Vitamin D Activation

Hess, Weinstock, and Helman (1) found increased transmission of u.v. irradiated cholesterol, correlated with activation. Hess and Weinstock (2) made more extensive absorption studies, and found that prolonged irradiation resulted in increased absorption. Schlutz and Morse (3) found bands in cod-liver oil at 3280 and 2790 Å., and bands in cholesterol at 2950 and 2810 Å. Irradiation of cholesterol caused the bands to disappear, but resulted in increased absorption in other regions. They suggested that the characteristic absorption might be due to a contaminant, but abandoned that theory later. Schlutz and Ziegler (5) found bands in hydrated but not in dehydrated cholesterol.

B. Ergosterol as the True Precursor of Vitamin D

Rosenheim and Webster (8) found by digitonin precipitation that but a small fraction of cholesterol is changed by u.v. irradiation. Rosenheim and Webster (9) concluded that the vitamin precursor is a contaminant of cholesterol, because cholesterol, purified through the bromide, could not be activated. Simultaneously, Heilbron, Kamm, and Morton (10) announced that the least soluble fraction from successive crystallizations of cholesterol from ethyl acetate showed more pronounced selective absorption, and deduced that the absorbing substance and provitamin are an identical contaminant of cholesterol. Pohl (11) came to similar conclusions by comparing the spectra of "ordinary" cholesterol, cholesterol purified by bromination, and irradiated cholesterol. Pohl (12) identified the contaminant with ergosterol on the basis of spectrographic observations. Windaus and Hess (13, 14) discuss the contaminant theory from the standpoint of the failure to activate brominated cholesterol, and cite experimental evidence for the extreme activatability of ergosterol. Rosenheim and Webster (15) deduced the amount of ergosterol present in cholesterol by spectrographic means, and found it of the proper amount to account quantitatively for the activatability of cholesterol. In a later paper (16) Heilbron, Kamm, and Morton discuss their fractionation experiments more fully. Rosenheim and Webster (18) give a more detailed account of their experiments and discuss the spectra of stigmasterol, sitosterol, etc. Jendrassik and Kemenyffi (19) express the opinion that the provitamin is formed naturally from cholesterol until an equilibrium is reached. Later (20) they identify the provitamin with ergosterol. Fabre and Simonnet (21) found zymosterol to be activatable, but their results may have been due to contamination with ergosterol. Bills, Honeywell, and MacNair (22), by comparing absorption photographs of ergosterol and cholesterol solutions, concluded that the ergosterol present is not sufficient to account for the activatability found by biological experiments. They associated further activatability with absorption bands at 3150 and 3040 Å. Heilbron, Morton, and Sexton (26) identified the 3150 and 3040 Å. bands of B., H., and M. (22) with those of the inactivatable cholesterilene. Kon, Daniels, and Steenbock (27) repeated the experiments of B., H., and M., (22), but decided that the conclusions of the former authors with regard to the lack of sufficient ergosterol in cholesterol to account for its activatability were due to inaccuracy of deductions from spectrum photographs. Kon (28) published similar material. Montignie (29) deduced the provitamin to be an isomer of ergosterol and cholesterol by associating phosphorescence with vitamin D. Koch, Koch, and Lemon (32) obtained by similar methods the same results as B., H., and M., (22) to the effect that ergosterol cannot account entirely for the activatability of cholesterol. Rosenheim and Webster (33) found Reindel's isoergosterol (34), and digitaligenin inactivatable. The same authors (39, 40), by parallel spectrographic and biological tests, concluded that the activatability of various sterols of ergot is due to traces of ergosterol.

INTRODUCTION

The purpose of this review is to outline the material that has been presented in the literature up to the present time regarding vitamin D and related subjects. In preparing it an attempt has been made to present all of the facts of importance in the various articles reviewed, so that one need not refer to the original articles except for secondary details. Such an ambitious scheme has resulted in a rather lengthy treatment, and has necessitated dividing the review into a number of parts, which will appear in the succeeding issues of this journal. Whether or not such an extensive program is advisable remains to be seen.

Part I, dealing with the relation of ultra-violet absorption spectra to the vitamin D problem, will be completed in this and the two following issues of the journal. Part II will deal with the artificial production of vitamin D—the historical background, the use of light, X-rays, etc., for vitamin D formation, the question of wave-lengths, the production of vitamin D in vivo, etc. Part III will discuss the assay of vitamin D, biological tests, color tests, etc. Part IV will be devoted to the chemistry of vitamin D.

HISTORICAL BACKGROUND

It was early realized that sunlight plays an important role in the prevention of rickets, a deficiency disease characterized by improper calcium metabolism. Trial of artificial light sources showed that it is the ultra-violet portion of the spectrum which is effective in preventing the disease.

The addition of cod-liver oil to the diet was also known to prevent rickets. It was natural to reason that the antirachitic activity of this dietary addition on the one hand, and of ultra-violet irradiation on the other, might be related in some way.

Consequently, substances of various kinds were irradiated with ultra-violet light and added to the diet of rachitic animals. Some of these successfully arrested or cured the disease; in particular, irradiated oils and fats were found to be powerful antirachitic agents. The unknown antirachitic factor produced by irradiation was given the name "Vitamin D."

Investigators then began to search for the parent substance of the vitamin in those irradiated oils and fats which had been used successfully. First, this precursor was found to be contained only in the unsaponifiable fraction of such materials. Later, it was found to be identified with the cholesterol content of the unsaponifiable fraction. Still later, it was deduced that a contaminant, ergosterol, and not the cholesterol itself is the true provitamin.

From the time that Vitamin D was first known to be formed photochemically by ultra-violet light, absorption spectra have played important roles in establishing the nature of the parent substance of the vitamin, in corroborating biological tests of vitamin D potency, and in shedding light upon the chemical nature of the active material—hence the choice of ultra-violet absorption spectra as the subject of the first part of the review.

ABSORPTION SPECTRUM TECHNIQUE

Various workers have used diverse methods both for recording and plotting the absorption spectra discussed herein. To save space, the technique used for determining the spectra, where reported, will be indicated by the code letters in Table I.

TABLE I

Technique For Determining Spectra

CODE	METHOD OR EQUIPMENT USED
A	Quartz spectrograph or monochromator (double prism train indicated by ^{2}).
B	Grating spectrograph.
a.	Mercury arc source.
b.	Tungsten spark source.
c.	Iron spark source.
d.	Under-water tungsten spark source.
e.	Hydrogen discharge tube source.
f.	Iron-nickel arc source.
1.	Absorption read directly with photoelectric photometer.
2.	Spectra photographed, no curves drawn.
3.	Spectra photographed, curves drawn by matching absorption spectra with comparison spectra, one or the other or both being exposed for different times.
4.	Spectra photographed through Nicol prism spectrophotometer—curves drawn by matching as in 3.
5.	Spectra photographed, curves determined with photodensitometer.
6.	Spectra photographed with sector photometer, and curves drawn by matching.
7.	Spectra obtained by determining thickness of solution for extinction

After determining the fraction of the light absorbed at different wave lengths in the ultra-violet, it is customary to convert the values into absorption coefficients or extinction coefficients before plotting curves of absorption. The absorption coefficient is the K of the exponent in the absorption equation:

$$I_x = I_o e^{-KCX}$$

where: I_x = intensity of light transmitted by material.
I_o = intensity of light incident on material.
e = base of Napierian logarithms.
K = absorption coefficient.
C = concentration (if absorbing material is in solution).
X = thickness of absorbing layer.

If Briggs logarithms (base 10) are used instead of Napierian logarithms (base e), the absorption term in the exponent is known as the "extinction" coefficient, viz.:

$$I_x = I_0 10^{-Jcx}$$

where J is the extinction coefficient.

If the concentration is expressed in gram molecules per liter and the cell thickness in cm., the values obtained are known as

TABLE II

Methods of Plotting Spectral Curves

CODE	METHOD OR UNIT USED
AA	Absorption Coefficients $\left(K = -\frac{1}{cx}\log_e\frac{I_x}{I_0}\right)$
BB	Extinction Coefficients $\left(J = -\frac{1}{cx}\log_{10}\frac{I_x}{I_0}\right)$
CC	Molecular Extinction Coefficients $\left(J' = -\frac{1}{cx}\log_{10}\frac{I_x}{I_0}\right)$ (c in gm. mols. per l., x in cms.)
DD	Logarithms of thickness for extinction.
EE	Logarithms of Extinction Coefficients.
FF	$\text{Log}_e\,(I_0/I_x)$ (values not divided by C and X)
GG	$\text{Log}_{10}\,(I_0/I_x)$ (values not divided by C and X).
aa	Concentration in g. per l. or mg. per c.c.
bb	Concentration in g. per c.c.
cc	Concentration in g. per 100 c.c., or per cent.
dd	Concentration in mg. per 100 c.c.
ee	Concentration in gm. mols. per l.
I	Thickness in mm.
II	Thickness in cm.
III	Thickness in inches.

"molecular absorption coefficients," or "molecular extinction coefficients," according to whether Napierian or Briggs logarithms are used.

Frequently the molecular weights of the materials used are not known and it is impossible to express the absorption in terms of molecular extinction coefficients. Even with known molecular weights, some have chosen to express the concentrations differently, so that one finds extinction and absorption coefficients plotted in terms of grams per liter or milligrams per c.c., grams per c.c., grams per 100 c.c., etc. The thickness, likewise, has been expressed in cm. in some instances and in mm. in other instances. For the sake of uniformity, we have, where possible, converted all values to grams per liter concentration and cms. cell thickness, showing as well the molecular extinction coefficients in many instances.[1] The values shown for oils are, where possible, extinction coefficients for 1 mm. layers of the oils.

The curves and tables are marked according to the code of Table II to show the units used by the various workers whose papers are reviewed.

A. EARLY ATTEMPTS TO CORRELATE ABSORPTION SPECTRA AND VITAMIN D ACTIVATION

The earliest mention of ultra-violet absorption spectra in relation to the activation of vitamin D seems to have been made by Hess, Weinstock, and Helman (1) in 1924. In a brief note, they state that phytosterol and cholesterol acquire the property of protecting against rickets when irradiated with ultra-violet light, and that cholesterol so irradiated shows decreased absorption and transmission of a wider range of the shorter ultra-violet.

Reasoning that ultra-violet absorption spectra are characteristic of chemical structure, and that changes in absorption spectra are indicative of chemical changes, Hess and Weinstock undertook an extensive study of absorption spectrum changes brought about in cholesterol, dihydrocholesterol, and dihydrophytosterol by irradiation with ultra-violet. This work was published in 1925 (2).

Spectra were photographed (Aa2) using 40 mm. thicknesses of cholesterol in alcohol, 2.0 g. per l.

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[1] Since Beer's law may not hold for all of the materials discussed, the actual concentrations used in the measurements are noted below the figures.

The cholesterol was irradiated with a water-cooled Kromayer quartz mercury arc, in alcohol solution, in water suspension, and in the dry state. The state of the irradiated material was found to make inappreciable difference in the type of absorption changes obtained or in the production of antirachitic potency.

INCREASED TRANSMISSION OF IRRADIATED CHOLESTEROL

Spectra were photographed immediately after each irradiation. For times of irradiation up to one hour, the absorption became less (Figure 1). Prolonged irradiation for a time of seventeen hours resulted, however, in a reversal of this change—an increase of absorption to a degree greater than that of the original cholesterol solution, with a shift of the absorption "limit" toward long wave-lengths.[1] The increased absorption was greater for cholesterol irradiated in a watery suspension than for that irradiated dry.

LOWERING OF M. P. BY IRRADIATION

Cholesterol irradiated seventeen hours became somewhat yellow in color and acquired a lower m.p. (one sample falling from 147° C. (uncorrected) to 135° C.).

Samples irradiated thirty hours or more became decidedly yellow and "opaque" to ultra-violet and blue radiations.

It is pointed out that the sequence of absorption changes (increased transmission at first, followed by decreased transmission) corresponds to the course of antirachitic potency, which increases during the first period of irradiation and later decreases.

FAILURE OF H_2O_2 TO ACCOUNT FOR CHANGES

Regarding the possibility that the production of hydrogen peroxide might account for the increased transmission, the authors say, "transmission proved to be greater in tests in which cholesterol was irradiated in alcohol for a period of 3 hours than when 1 c.c. of 0.001 or 0.00001 normal hydrogen peroxide solution had been added. In these tests, as in some others, the

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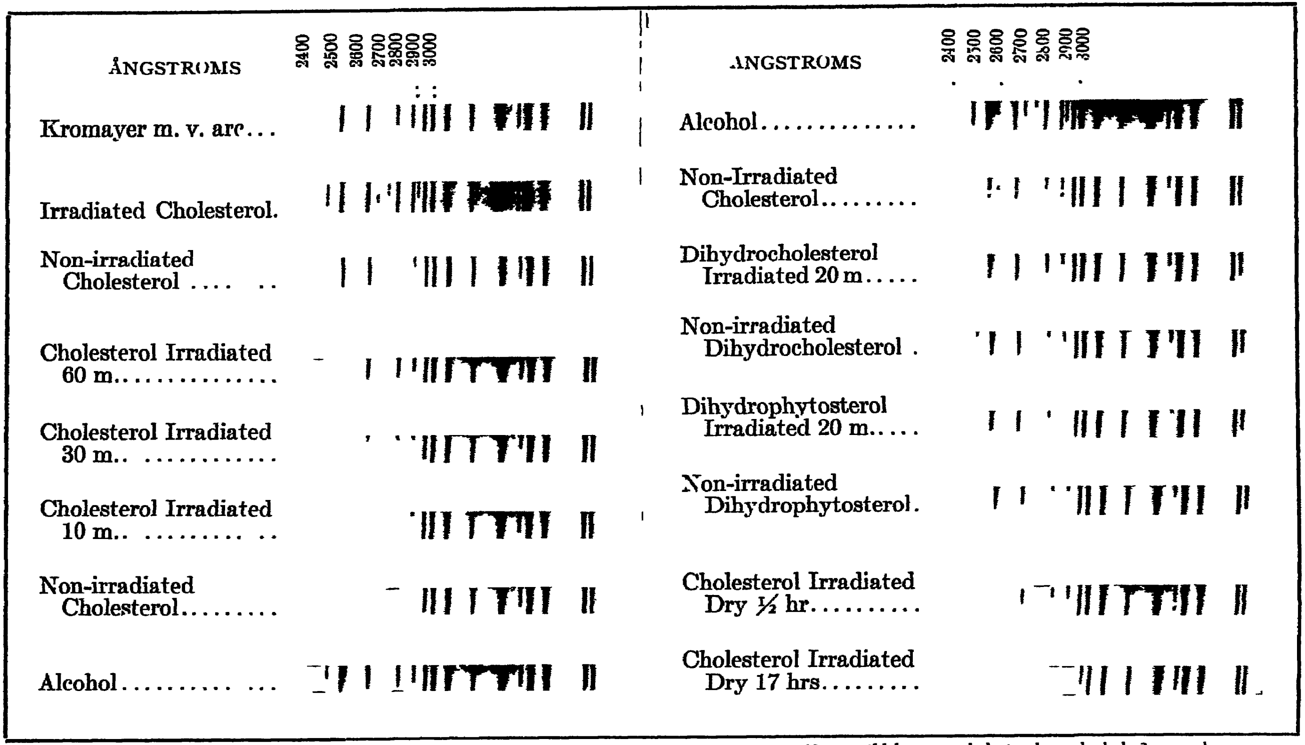
[1] The authors refer only to increased or decreased transmission. The shift in the absorption "limit" is our own observation from their plates.



40 mm. thicknesses cholesterol in alcohol, 2g. per l.

Fig. 1—Absorption of Cholesterol, Dihydrocholesterol, and Dihydrophytosterol [Hess and Weinstock (2; 1925)]

irradiated cholesterol transmitted the light rays even better than the 95 per cent alcohol." One wonders if this greater transparency than alcohol was actually due to fluorescence of the cholesterol. Both cholesterol and ergosterol have been shown to be fluorescent in ultra-violet light, as we shall see.

INERTNESS OF DIHYDROCHOLESTEROL AND DIHYDROPHYTOSTEROL TO RADIATIONS

Biological experiments had previously demonstrated irradiated dihydrocholesterol and dihydrophytosterol to be inactive antirachitically. Spectrograms showed that their absorption spectra were but little changed by irradiation. Such change as was produced was in the direction of increased absorption (Figure 1).

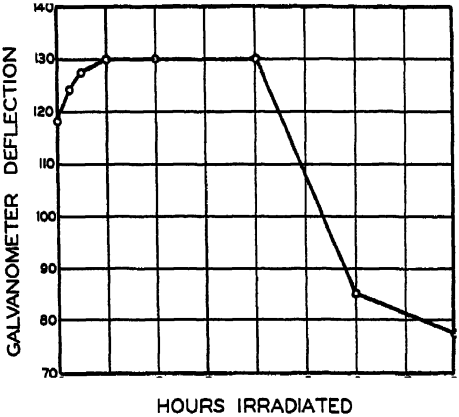


Fig. 2.--Total Ultra-Violet Transmission of Cholesterol Solutions Irradiated with Ultra-Violet for Various Periods [Hess and Weinstock (2; 1925)]

As a parallel check of the absorption changes of cholesterol occuring during irradiation, the total ultra-violet transmission of a cholesterol solution was determined with a copper-constantan thermopile and a galvanometer at intervals during irradiation (Figure 2).

RELATION OF LUMINESCENCE TO ACTIVITY OF COD-LIVER OIL

At this time (1924-25) the question as to how the cure of rickets by direct irradiation was related to its cure by cod-liver oil engaged the attention of various workers. Schlutz and Morse (3; 1925) believing the explanation to lie in the emission of radiant energy by cod-liver oil, repeated certain experiments of Kugelmass and McQuarrie (4), but failed to obtain blackening of photographic plates by the oil under various conditions, as reported by the latter authors.

They then turned their attention to absorption spectra, believing that "perhaps a key to the solution might be found by studying the absorption spectrum of cod-liver oil and comparing it with that of cottonseed oil, which has no antirachitic properties." They reasoned that: "when a substance exhibits a characteristic absorption band, it means that it can absorb energy at definite wave-lengths and this energy may be stored up and either given out again or used up in some way. The effect of the energy given out by the cod-liver oil might then be similar to the effect of the energy of the ultra-violet radiation. If the cod-liver oil showed an absorption spectrum characteristically different from that of cottonseed oil, and especially if the wave-lengths absorbed corresponded to the wave-lengths of the ultra-violet which are effective in curing rickets, this would be strong support for such a theory."

One knows that when radiations are absorbed by a material their energy is usually changed to another form. It may, for example, be converted into heat, or changed to chemical energy because of the formation of new chemical substances, or be re-emitted as fluorescent radiant energy of a wave-length different from that of the incident energy. Only in cases of reflection and transmission and in certain infrequent instances of fluorescence or phosphorescence does the incident energy reappear in its initial form, however, and then only in much reduced intensity. With these facts in mind, it is curious that the photochemical point of view should have been lost sight of by these and other early workers in the attempt to make cod-liver oil a mere physical storehouse, re-emitting ultra-violet rays known to be curative. The present viewpoint, that a chemical substance in the cod-liver oil is responsibile for curing rickets and that the same substance is produced in the body by ultra-violet light, seems much simpler.

ABSORPTION BANDS IN COD-LIVER OIL

Comparing cod-liver oil and cottonseed oil (A 7), Schlutz and Morse found two shallow absorption bands for cod-liver oil at about 3280 and 2790 Å. but only general absorption for cottonseed oil. The total ultra-violet absorption of the cod-liver oil was much greater than that of cottonseed oil, a 1.5 mm. layer of the former sufficing to absorb all radiations of wave-length shorter than 3770 Å. whereas layers of the latter at least twenty-four times as thick were required.

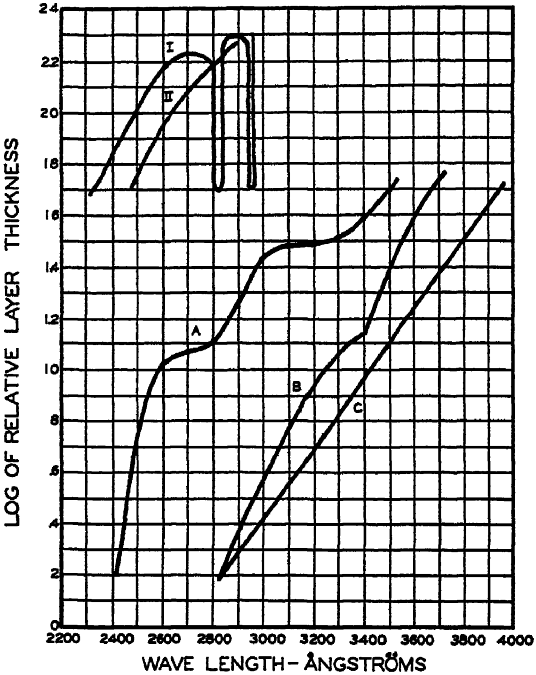
Since cod-liver oil is a mixture containing considerable fat with small portions of other substances, and since the shallow absorption bands found in it might be due to an active substance the characteristic absorption of which was masked by the general absorption of the oil, Schlutz and Morse thought it advisable to investigate the absorption of the unsaponifiable fraction of the oil, which had at that time already been demonstrated to be antirachitic. They obtained the unsaponifiable fraction as a cream-colored, semi-solid substance. Its spectrum was determined in two per cent absolute alcohol solution. The cholesterol, believed to be inactive, was then removed as well as possible from the unsaponifiable fraction by crystallization from alcohol, and the absorption of the residue examined in two per cent alcohol solution. For comparison, the absorption of cod-liver oil in alcohol in the same concentration was determined (Figure 3). The absorption bands of cod-liver oil appeared in its alcohol solution, but the unsaponifiable fraction, whether free from cholesterol or not, showed only general absorption.

CRITICISM OF THE FINDINGS OF HESS ET AL..

After their work had proceeded to this point, the work of Hess and others appeared (1924), in which it was stated that cholesterol and phytosterol become antirachitic upon exposure to ultra-violet light; and also the statement of Hess (1; 1924) that irradiated cholesterol becomes more transparent to ultra-violet radiations.

According to Schlutz and Morse, this latter statement of

Hess was to the effect that "spectrograms of cholesterol after irradiation transmit more of the extreme ultra-violet than before." Schlutz and Morse take exception to the idea that the transmission increases in the extreme ultra-violet, but we have been unable to find mention of the word "extreme" in the reports of Hess and his co-workers.



A, B, C, 2% solutions in absolute alcohol,
I, II, 4% solutions in ether, (DD)

Fig. 3—Absorption of Cod-Liver Oil Fractions and of Cholesterol [Schlutz and Morse (3; 1925)]

Curve *A*, cod-liver oil; curve *B*, unsaponifiable fraction of cod-liver oil; curve *C*, unsaponifiable fraction with cholesterol removed; curve *I*, cholesterol before irradiation; curve *II*, cholesterol after irradiation (5, 10, or 20 minutes, all the same).

Interpreting the increased transmission of the irradiated cholesterol of Hess et. al. to be the direct opposite of the increased absorption they had obtained for the fractionated active portions of cod-liver oil compared with the oil itself (why they should have compared the results of these totally different procedures is not clear), Schlutz and Morse repeated the work of Hess with cholesterol. They used Pfanstiehl's chemically pure cholesterol, crystallized twice, with a final m.p. of 148.5° as compared with 143° for the original material. Evaporation of the mother liquor of the last recrystallization gave a product with a m.p. of 148.1°.

ABSORPTION MAXIMA OF CHOLESTEROL

Testing the absorption of this purified cholesterol in 1 and 2 per cent absolute alcohol solutions they found "that absorption due to the effects of the alcohol concealed the effects due to the cholesterol."[1] Abandoning alcohol as a solvent, they used ether dried over calcium chloride and distilled, which, they say, is considerably more transparent in the shorter ultra-violet. In a 4.2 cm. layer of 2 per cent solution they found little general absorption and two faint bands. A 4 per cent solution, layers up to 9.1 cm., gave more definite bands, with maxima at 2950 and 2810 Å. and, of course, an increased general absorption. As we shall see, the maxima were probably ergosterol bands.

INCREASED ABSORPTION OF IRRADIATED CHOLESTEROL, CONTRARY TO FINDINGS OF HESS ET. AL.

Ether solutions of cholesterol were irradiated with a quartz mercury arc for times of five, ten, and twenty minutes, and their concentrations readjusted after irradiation. Spectrographic examination of the irradiated solutions showed that the characteristic absorption bands had disappeared but the general absorption had increased (Figure 3). The absorption had decreased in the 2800-2950 Å. region, because of the degradation of the bands, but had increased throughout the region shorter than 2780 Å. One will note from an examination of Figure 1 that Hess and Weinstock (2) obtained increased transmission even at 2520 Å.

Commenting upon this, Schlutz and Morse say, "Our results do not agree with the findings of Hess that the irradiated cholesterol transmits more of the ultra-violet, for in every case general absorption in the irradiated cholesterol cuts off more of the ultra-violet than in the cholesterol which has not been irradiated, that is, the spectrum through corresponding layer thicknesses was visible further out toward the short wave-lengths of the ultra-violet in the case of the non-irradiated cholesterol. However, it is possible that Hess was referring to the region in which selective absorption takes place in the cholesterol. In that region it is true that the irradiated cholesterol transmits more, but that region could hardly be called the extreme ultra-violet."

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[1] One must realize that they were using a long (adjustable) cell. Consequently the absorption of the solvent was appreciable.

POSSIBLE EXPLANATION OF THE DISCREPANCY

In comparing these inconsistent results one must realize that neither group of workers specifies the intensity of radiations used, or even the distance from the lamp. It is possible that the intensity was so much greater in the experiments of Schlutz and Morse that all of their samples correspond to over-irradiated samples of Hess and his associates, or perhaps different spectral energy distributions in the two cases may account for the non-concordant results.

The curves of Figure 3 were plotted by obtaining the layer thicknesses at different wave-lengths corresponding to "limits of absorption." Realizing the inaccuracy of such a method,[1] the authors plotted other curves by means of a densitometer, with equal thicknesses of cholesterol solution irradiated and un-irradiated (for these curves, refer to the original article). The published curves show definite bands at about 2820 and 2930 Å. which disappeared upon irradiation, and an increased absorption of the irradiated material in the region shorter than about 2680 Å., but a decreased absorption in the 2680-2860 Å. region. These results, at least as we interpret them, are in somewhat better agreement with the findings of Hess and Weinstock (2).

THE ANTIRACHITIC FACTOR IN COD-LIVER OIL

"Examination of the graph shows," Schlutz and Morse say, "that if the irradiated cholesterol is the same substance as the antirachitic factor in cod-liver oil, then it could not show up in the absorption spectrum of cod-liver oil, for it is so little absorptive and its concentration is so small. The absorption of the oil and its unsaponifiable fraction obtained must then be due to some constituent of the oil other than the active factor. A problem still to be solved is whether the antirachitic substance in the cod-liver is the same as that which is formed when cholesterol is irradiated."

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[1] There is no true "limit of absorption," there being some transmission for all layers of finite thickness. The method used here consists simply in increasing the cell length until the transmission is too low to give a noticeable blackening of the photographic plate under the conditions of the experiment. Such a method is accordingly inaccurate.

Their spectrograms of cholesterol showed disappearance of the characteristic bands during the first five minutes of irradiation but no further change in absorption up to twenty minutes. Since Hess and Weinstock (2) obtained increasing transmission (probably due to the disappearance of the selective absorption bands) up to one hour irradiation (Figure 2), it is probable that the intensity used for irradiation by Schlutz and Morse was greater than that employed by Hess and Weinstock.

IS THE CHARACTERISTIC ABSORPTION DUE TO A CONTAMINANT?

Since layer thicknesses of 7 cm. failed to give the bands after irradiation, which were easily detectable in 3.2 cm. thicknesses of the non-irradiated material, they concluded that at least half of the cholesterol had been changed to some other form. They point out, however, another possibility—that the substance responsible for the characteristic absorption band might be an impurity contaminating the cholesterol. This impurity might be completely transformed without the cholesterol being changed. In this statement, based on spectrographic evidence, one finds the first suggestion that a contaminant rather than cholesterol itself might be the true precursor of vitamin D.

LOWERING OF M. P. BY IRRADIATION CONFIRMED

In agreement with Hess and Weinstock (2), the m.p. of cholesterol was found to change upon irradiation. Samples originally melting at 148.5° gave a residue after irradiation and evaporation from the solution which melted at 143°. Dissolving cholesterol in ether and recovering it by evaporation of the solvent produced no change in the m.p. When the irradiated ether residue was recrystallized from alcohol, however, there resulted a large crop of unchanged cholesterol crystals, m.p. 148.5°. On evaporation of the mother-liquor, a small residue was obtained which "apparently contained more cholesterol but had a brownish color." The residue started to clump together at 135°. From this they deduced that but a small amount of the cholesterol had been changed chemically by irradiation, unless it were possible that solution in hot alcohol reconverted the irradiation product into cholesterol.

SCHLUTZ AND MORSE ABANDON THE CONTAMINANT THEORY

Having actually arrived at the truth in suggesting the possibility of the characteristic absorption being caused by a contaminant of cholesterol, changed by irradiation, they now abandon this point of view in considering the result of recrystallization from alcohol. "It seems reasonable to correlate all of these facts," they say, "by the supposition that the whole of the cholesterol is converted by irradiation into a different, more active form which is unstable and which is destroyed by evaporation of the solvent, or by solution in alcohol. The lowering of the melting point may be explained by the formation of another product through a secondary reaction, a supposition which is borne out by the fact that Steenbock has shown that prolonged irradiation of cholesterol destroys its antirachitic activity entirely."

Regarding the identity of the active substance in cod-liver oil with activated cholesterol, they say that, aside from the problem of how cholesterol in the fish became activated, the different stability of the two causes a difficulty in correlating them. They point out that the active factor in cod-liver oil is reported as being quite stable, resistant to boiling in concentrated alcoholic alkali during saponification, and stable during the crystallizing out of the cholesterol. "On the other hand," they say, "the activated cholesterol material (as we have seen) apparently is converted to inactive cholesterol by the simple process of evaporating the ether and recrystallizing from alcohol."

At this point they fail again to consider the possibility they have themselves suggested that the active material might be a contaminant of the cholesterol rather than an active form of cholesterol itself.

In conclusion they suggest that the disappearance of the selective absorption bands in the spectrum of cholesterol be used as an index of the degree of activation, thereby eliminating the necessity of animal experiments, a scheme which was to be attempted by many workers later.

SELECTIVE ABSORPTION AND PHOTOCHEMICAL CHANGE

Somewhat later, Schlutz and Ziegler (5; 1925) retaining the viewpoint that only radiations absorbed at the wave-lengths corresponding to *selective* absorption are responsible for activation, undertook an investigation of the two selective absorption bands found in the spectra of cholesterol by Schlutz and Morse (3).

Again and again in this review we shall see this viewpoint motivating investigations. Few workers seem to associate photochemical with photoelectric effects, and few, therefore, embrace the quantum viewpoint that photochemical effects begin at critical wave-lengths in the spectrum and continue for shorter wave-lengths, whether absorption is "general" or "selective" in the active region. The quantum viewpoint, which seems to explain the facts of ergosterol activation better than any other, is well stated by Schneider and Sperti (6; 1926), and Shepard (7; 1929).

Schlutz and Ziegler first compared two cholesterol preparations, one from cod-liver oil and the other from Pfanstiehl's cholesterol, both recrystallized twelve times in absolute alcohol and separated into five fractions. The purest fractions of the two cholesterols gave identical color reactions, melting points, and absorption spectra; from which they deduced that the two cholesterols were identical, and that the cod-liver oil preparation could not be contaminated with a substance other than cholesterol carried over from the oil. Thus, they had, to their satisfaction, eliminated again the possibility of a contaminant of cholesterol being responsible for the selective absorption spectrum. Both samples showed the two absorption bands ("between 2940 and 2960 Å. and between 2790 and 2940 Å.") previously obtained. Their melting points were 148.5° C.

MAXIMA IN HYDRATED BUT NOT IN DEHYDRATED CHOLESTEROL

They then compared the spectra (Ad) of hydrated cholesterol (containing one molecule of water), m.p. 148.5° C., and the anhydrous form, m.p. 147.0° C. By examination of a number of samples, they found that all which had melting points of 148.5° C. or thereabouts showed the characteristic bands, while those which melted below this point either showed no bands or very weak ones.

Dehydrated cholesterol samples prepared by treating hydrated cholesterol in an oven at 110° C., by crystallization from ether or chloroform, by precipitation from glacial acetic acid, by fractional crystallization with alcohol of samples dried at 100° C., and by sublimation in vacuo below 110° all failed to show the characteristic bands.

If cholesterol, m.p. 148.5° C., was allowed to stand in solution in anhydrous ether, there resulted a disappearance of the bands[1] and a lowering of the m.p. to 147.0° C. When the recovered cholesterol, m.p. 147.0° C., was dissolved in ether, it gave the "typical short spectrum with no bands and considerable general absorption." Treating this dehydrated sample with water twice raised the m.p. to 148.5° C., and resulted in reappearance of the bands.

An attempt is described to determine whether or not the bands were obscured by general absorption, by varying the concentration, cell thickness, and time of exposure. One is mainly impressed in this discussion with the great difficulty they had in obtaining bands under any conditions—even with the cholesterol of m.p. 148.5° C.

Irradiation of dehydrated cholesterol in ether solution for six hours at one foot from a mercury arc resulted in only a slight increase of absorption. Similar treatment of hydrated cholesterol showed considerable increase in absorption.

Upon photographing spectra of hydrated cholesterol in anhydrous alcohol, they found no bands. In chloroform, hydrated cholesterol gave bands, but the anhydrous did not, as in the case of the ether solvent. Pentane was tried but did not dissolve enough cholesterol to be used satisfactorily.

They conclude that hydrated cholesterol has two characteristic absorption bands at about 2940 and 2790 Å., and that anhydrous cholesterol has no characteristic absorption bands but much greater general absorption.

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[1] This change in absorption on standing, observed by several workers, will be discussed in greater detail later.

No absorption spectra are reproduced in the paper.[1]

B. ERGOSTEROL AS THE TRUE PRECURSOR OF VITAMIN D

Up to this time, interest in ultra-violet absorption spectra had been concentrated upon the study of the spectra of cholesterol and of various activatable oils in order to show the presence of cholesterol as the activatable factor in these oils; and on the absorption changes occuring in cholesterol during irradiation. Experimenters now began to turn their attention to the separation of the active fraction from irradiated cholesterol, and to the determination of the types of linkages in the sterol molecule necessary for activatability.

EVIDENCE OF A CONTAMINANT OF CHOLESTEROL

A number of anomalies presented themselves. Schlutz and Morse (3) had found irradiation of cholesterol characterized by a rapid disappearance of selective absorption, with little change thereafter, and had at one time suggested that the selective absorption might be due to a contaminant of cholesterol.

Rosenheim and Webster (8; 1926) found that when cholesterol was irradiated in an atmosphere of nitrogen and the unchanged material precipitated by digitonin, but a fraction of a per cent of the material failed to be precipitated, indicating that only a minute amount of the supposedly pure cholesterol had been changed by irradiation.[2] Animal experiments showed the unprecipitated fraction to be protective.

In these findings we have the first definite indications that cholesterol is at least not the only precursor of vitamin D and perhaps not the true precursor at all.

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[1] Consequently one hesitates to review the results critically. It is difficult to judge without seeing the evidence. Your reviewer, however, believes it quite possible that failure to find bands in the dehydrated samples was due to their increased general absorption and "typical short spectrum." The bands are none too prominent in spectrum photographs of ordinary cholesterol. It is easy to pass them over by failure to choose just the proper combination of exposure time, concentration, and cell thickness. With increased general absorption to obscure them, it would be even easier to miss them—in fact it might be impossible to detect them by the crude method of superficial examination of spectrum photographs apparently used by the authors.

[2] The authors, however, do not suggest this conclusion.

Continuing their investigations, Rosenheim and Webster (9; 1926) came to the conclusion that it is not cholesterol itself but a substance associated with it which is capable of activation. This paper was read before the Biochemical Department of the Imperial College of Science and Technology, London.

INACTIVATABILITY OF BROMINE-TREATED CHOLESTEROL

By converting "pure cholesterol" to the dibromide and reducing the latter with sodium amalgam to cholesterol, they found that the cholesterol thus purified no longer became antirachitic when irradiated. They state that Professor Windaus prepared a similarly purified sample, at their suggestion, and found that it no longer showed the absorption spectrum in the ultra-violet characteristic of so-called "pure" cholesterol.

Rosenheim and Webster concluded that the precursor is a substance associated with cholesterol which follows it through all the stages of purification by the usual methods of saponification and recrystallization, and they proposed to call this precursor "vitasterol."

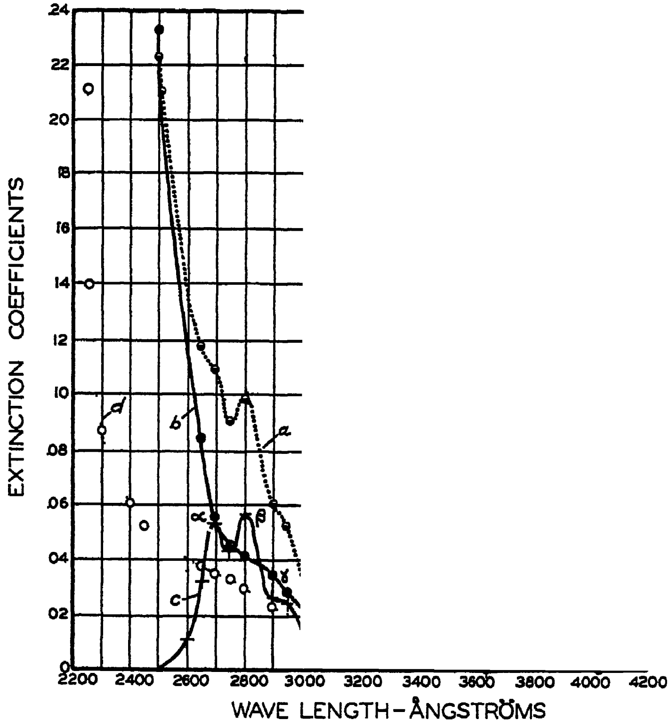
INCREASED SELECTIVE ABSORPTION OF CHOLESTEROL FRACTIONS

At the same meeting, Heilbron, Kamm, and Morton (10; 1926) presented a paper in which they stated that by fractional crystallization of cholesterol they had concentrated a substance in the least soluble fraction which had properties different from the non-fractionated material. They found that highly-purified cholesterol had only general absorption in the ultra-violet, while the new substance had three well-defined absorption bands at 2930, 2800, and 2690 Å. Reference is made to the work of Schlutz and Ziegler (4), who, it will be remembered, found selective absorption for cholesterol between 2940 and 2960 A., and between 2790 and 2940 Å.

The characteristic bands of the concentrated fraction disappeared when the material was irradiated with ultra-violet light, either in the solid state or in solution in alcohol or ether. Since such irradiation was known to induce antirachitic potency in cholesterol, they deduced that there was a strong evidence "for a correlation between the substance showing the absorption bands and the vitamin precursor."

THE PROVITAMIN A CONTAMINANT OF CHOLESTEROL

Thus, to Schlutz and Morse we owe the first suggestion that a contaminant of cholesterol is the true provitamin, and to Heilbron, Kamm, and Morton, and to Rosenheim and Webster, jointly, we owe the first experimental proof of this viewpoint.



1% solutions in alcohol (AAccI)

Fig. 4—Absorption of Cholesterol [Pohl (11; 1926)]

Curve *a*, ordinary cholesterol; curve *b*, cholesterol irradiated 20 m.; curve *c*, difference between curves *a* and *b*; curve *d*, cholesterol purified by bromination.

Pohl (11; 1926) became interested in the changes in the absorption spectrum of "cholesterol" upon irradiation, as reported by Hess and Weinstock (2; 1925), because he and Gudden had at one time conducted an investigation of the changes in absorption spectra brought about in certain crystals by irradiation, a phenomenon which they did not believe to be a photochemical reaction in the usual sense but rather an "electron displacement." Pohl felt that perhaps the changes brought about in cholesterol by irradiation might be of a similar character.

He and his assistants investigated the absorption spectra (A-1) of preparations supplied by Professor Windaus. Absorption through 1 per cent solutions of the preparations in alcohol were compared with the absorption of a similar cell containing the solvent. A cholesterol specimen recrystallized six times from alcohol showed the spectrum illustrated in Figure 4, *a*. Observations made by two assistants with different equipment are said to "agree very well." Curve *a* has an inflection at about 2700 Å., a peak at about 2800 Å., and an inflection at about 2900 Å.

ABSORPTION CHANGES OBTAINED BY POHL

After irradiation for twenty minutes with the light from a magnesium spark, this preparation gave curve *b*. A magnesium spark was chosen for irradiation because of its strong line at about 2800 to 2850 Å. which "would appear from the work of Hess and Weinstock to be especially suited to change cholesterol into the antirachitic form." Pohl states that the spark causes as great a change in ten minutes as can be obtained with the full radiation from a mercury arc in one hour, the mercury arc having no intensive emission lines between 3000 and 2540 Å. A further advantage of the magnesium spark, he says is that one need take no precautions against warming the cholesterol solution during irradiation.

Observation of curve *b* shows that the effect of irradiation has been to increase considerably the transmission from about 2500 to 3100 Å. in agreement with the findings of Hess and Weinstock (2). To show the nature of the absorption changes better, the extinction coefficients of curve *b* were subtracted from those of curve *a*, resulting in curve *c*. Pohl says, "It is seen that by irradiation of the cholesterol preparation a band system has disappeared in which three maxima, α β γ, may well be real, while the maximum δ must still be regarded as questionable." The spectrum of the substance destroyed by irradiation which he thus obtained is strikingly like that of ergosterol.

CONCLUSION FROM THE ABSORPTIVE CHANGES THAT THE PROVITAMIN IS A CONTAMINANT

He points out that the absorption coefficient at 2800 Å. has been decreased by irradiation from 0.022 to 0.01,—more than half.[1] If the view of Hess and Weinstock (2) were correct, that this absorption spectrum is characteristic of cholesterol, and that coincident with the decrease in absorption the cholesterol is transformed photochemically into an antirachitically active substance, then this change in extinction coefficient would indicate that about half of the original cholesterol had been changed. This, Pohl says, is not consistent with the chemical results of Windaus, who found that at least 99.5 per cent of the original cholesterol remains unaltered by irradiation. The answer to this inconsistency, he concludes, can only be that the characteristic absorption spectrum which disappears upon irradiation is not due to cholesterol itself but to some other material which acts as a "dyestuff" in the sense that in minute quantities its presence in cholesterol is sufficient to account for the increased absorption.

If it were true that this characteristic absorption were due to a contaminating substance, it should be possible to prepare cholesterol free from these bands, and Pohl states that Windaus had succeeded in obtaining a series of cholesterol preparations in which the bands were scarcely if at all present. As an example, he cites curve *d*, which is of a cholesterol preparation purified twice by bromination. The characteristic bands are almost entirely absent, and irradiation was found to be ineffective in changing the absorption spectrum.

Preparations of cholesterol obtained by adding and splitting off HCl, and of sitosterol purified through the bromide, both prepared by Professor Windaus, also showed this lack of selective absorption and inertness to irradiation.

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[1] These values are in terms of $\text{Log}_e\ (I_0/I_\lambda)$, 1 % solutions, 1 mm thickness The corresponding values as we have plotted them here ($\text{Log}_{10}\ (I_0/I_\lambda)$, concentration 1 g. per 1., 1 cm. thickness) are 0.095 and 0.043, respectively.

Pohl concludes that the substance having a characteristic absorption spectrum, and being at the same time the vitamin precursor, is an impurity in cholesterol. "As yet one has not determined what this impurity is chemically. Still one may use the characteristic absorption as an indication of its amount, just as one uses the emission of radioactive substances as an indication for amounts of materials too small to be determined chemically."

This paper is dated December 10, 1926, just four days after the papers of Rosenheim and Webster (9) and Morton, Heilbron, and Kamm (10) had been presented.

ABSORPTION EVIDENCE THAT THE PROVITAMIN IS ERGOSTEROL

A second paper by Pohl (12), presented January 14, 1927, gave evidence that the contaminant responsible for the selective absorption of cholesterol is ergosterol.[1]

The absorption of ergosterol was examined (A^21) in 0.001 per cent solution in alcohol and compared with that of a 1 per cent antirachitically activatable cholesterol solution in alcohol (Figure 5). The positions of the absorption peaks were found to be identical.

The absorption [$\text{Log}_e\ (I_0\ I_x)$] of the 0.001 per cent ergosterol solution peaks was about six times as great as that of the 1 per cent cholesterol solution peaks, from which it was deduced (assuming that the absorption of cholesterol was due to ergosterol) that the cholesterol was contaminated with ergosterol to the extent of about 0.016 per cent (1 part in 6000). Assuming the ergosterol only to be changed by irradiation, this confirmed Windaus's findings that but minute amounts of his cholesterol preparations were changed chemically during photoactivation.

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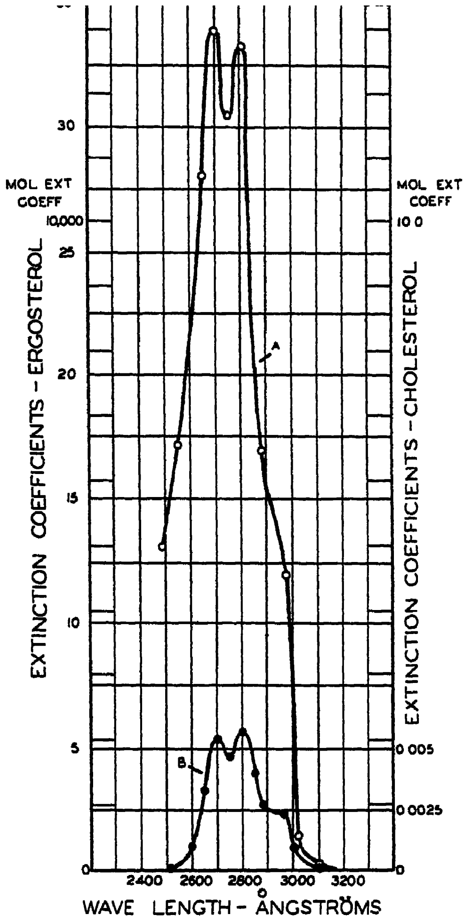
[1] A report of a speech by Pohl in which he outlined his experiments is given in another journal (42; 1927).

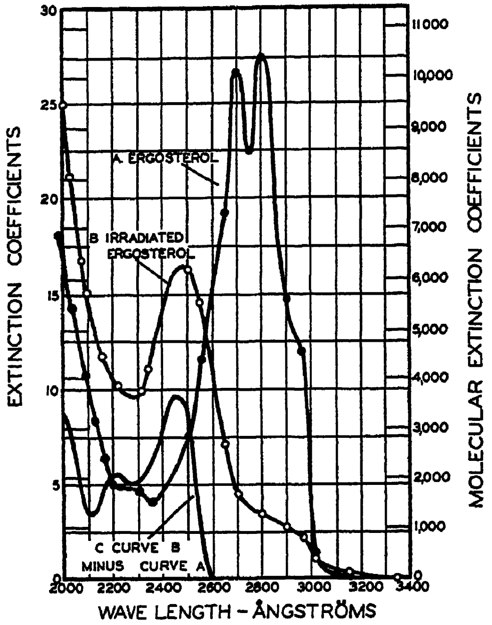
A few minutes' irradiation of ergosterol solutions with a magnesium spark gave a clear spectrum change, characterized



A, 0.001 per cent in alcohol, B, 1 per cent in alcohol (FF)

Fig. 5—Absorption of Ergosterol and Cholesterol [Pohl (12; 1926)]
Curve *A*, ergosterol; curve *B*, cholesterol.

by a fading of the band near 2800 Å. Curves of unirradiated ergosterol (A) and ergosterol irradiated 25 m. with the magnesium spark (B) are shown in Figure 6. The difference between these curves is said by Pohl to represent the absorption spectrum of the vitamin (Figure 6, C).[1]



0.04 per cent solutions in alcohol (FF).

Fig. 6—Absorption of Ergosterol

Curve A, unirradiated ergosterol; curve *B*, ergosterol irradiated 25 m.

Windaus and Hess (13; 1927) describe attempts to determine the nature of vitamin D on the assumption that it is a photochemical transformation product of cholesterol. In the course of this work, methods of preparing various plant sterols through their bromine addition products were worked out, and it was found that specimens of cholesterol and other sterols so purified could not be activated. The researches of Rosenheim and Webster, Heilbron, Kamm and Morton, and Pohl—with whom these authors worked closely—are described, and the evidence for provitamin D being a contaminant of ergosterol is discussed. Irradiated ergosterol was found to have high antirachitic potency, and the addition of small quantities of ergosterol to brominated cholesterol was found to lead to a product having the biological and other properties of "ordinary" cholesterol.

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[1] "The positive difference between the two curves . . . shows the absorption newly produced by the photochemical change. . . . It is accordingly the spectrum of the photochemical reaction product or of the vitamin. It lies more prominently in the short-wave region than ergosterol or the provitamin. Further measurements will clear up certain considerations, for example whether there exist weaker bands in the long-wave region." Actually the method used is not exactly legitimate for obtaining the true absorption of the reaction product. To obtain the actual absorption, one must know the fraction of unchanged ergosterol present in the irradiated material, and must multiply the values of curve *A* by this fraction before subtracting them from those of curve *B*.

Similar experiments (inactivatability of brominated cholesterol and phytosterol, etc.) are described by Hess and Windaus (14; 1927).[1] It is stated that over-irradiated cholesterol which had been recrystallized could not be activated. The authors suggest that the provitamin is a contaminating substance present in cholesterol.

OTHER BROMINE-TREATED STEROLS INACTIVATABLE

Rosenheim and Webster (15; 1927), in coöperation with Windaus, found that phytosterol as well as cholesterol failed to be activatable after having been purified through bromination and by other means.

At Professor Heilbron's request, they tested two cholesterol fractions prepared by Heilbron, Morton, and Kamm (10) which had absorption values four times as great as those of ordinary cholesterol. Their biological tests indicated that the provitamin content agreed with this increased absorption. In this connection they state that "we are further informed by Professor Windaus that he has found it possible, by fractional distillation of cholesterol in a high vacuum, to produce a nine to ten fold increase in provitamin content as measured by the optical test."

Various indications pointed to ergosterol as the possible provitamin contaminant of cholesterol (we have seen how

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[1] A report of a speech by Windaus in another journal (43; 1927) outlines the same material briefly.

Pohl had already arrived at that conclusion). From the absorption standpoint, Rosenheim and Webster say, "from the fact that ergosterol possesses three unsaturated linkages, one would expect it to show a greater absorption in the ultra-violet region than cholesterol, which has only one double bond." They therefore took up again, at Professor Windaus' suggestion, the study of ergosterol, which they previously (8) shown to be highly antirachitic after irradiation with ultra-violet.

Following the indication of the work of Heilbron, Kamm, and Morton (10) and of Pohl (11) they compared the absorption of ergosterol with that of a specimen of "ordinary" cholesterol. They found that ergosterol had great absorption in the ultra-violet, "the characteristic band of absorption being like that of cholesterol at 2800 Å., but of enormously greater intensity." They estimated the intensity of ergosterol absorption as 1500 to 2,000 times that of brain cholesterol.

Upon irradiating ergosterol, the characteristic absorption band disappeared (in agreement with Pohl, 12), as in the case of the cholesterol used in previous experiments. At the same time, the product of irradiation lost its property of being precipitated by digitonin.

ESTIMATES OF THE ERGOSTEROL CONTENT OF CHOLESTEROL

From the comparative absorption intensities they deduced the amount of ergosterol present in cholesterol to be of the order of one part in 2,000, or 0.05 per cent. If this were true, the protective dosages of irradiated ergosterol should be of the order of 1/2000 those of irradiated cholesterol. Biological tests confirmed these assumptions, daily dosages of 4/1000 mg. of irradiated ergosterol curing and preventing rickets, and tests with still smaller quantities "not yet completed," indicating a limit of activity of less than 1/1000 mg. This compares with 4 mg. per day of irradiated cholesterol found by Rosenheim and Webster in previous work to be completely protective, and 0.2 mg. per day, found by them to be the limit of protection.

Rosenheim and Webster conclude that the parent substance of vitamin D "if not ergosterol itself is a highly unsaturated sterol of similar constitution." These experiments were carried out in coöperation with Professor Windaus.

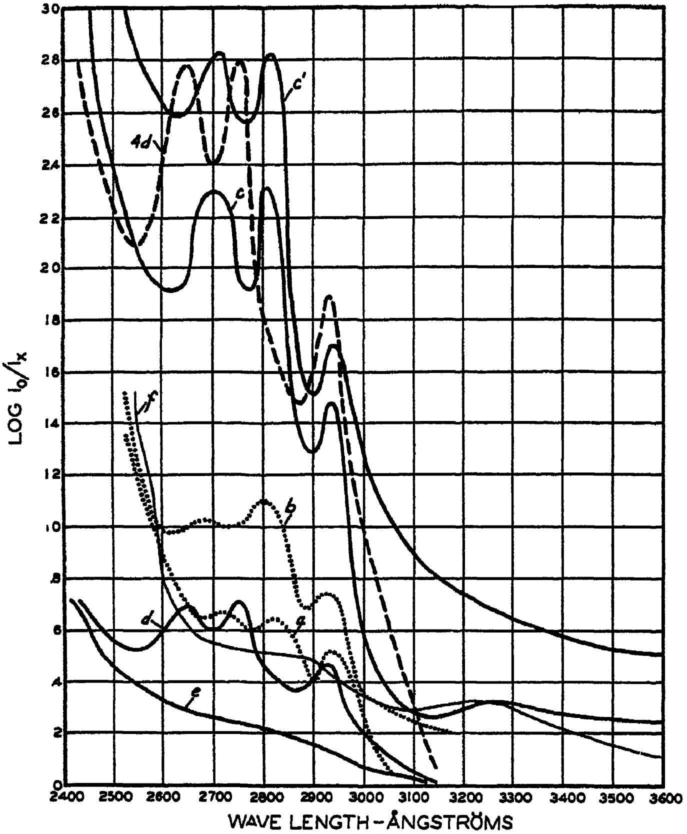
FURTHER WORK ON FRACTIONATION OF CHOLESTEROL

The experiments of Heilbron, Kamm, and Morton in fractionating cholesterol are discussed in somewhat greater detail in a later paper (16; 1927). They suggest that the absorption obtained by Hess and Weinstock (2) and Schlutz and Ziegler (5), for cholesterol on the one hand, and the extreme light sensitiveness and activatability of provitamin D on the other, makes it inconsistent to regard cholesterol as provitamin D. Here we have new considerations regarding absorption entering into the question of the vitamin precursor. They were led by these considerations to undertake a quantitative study of the ultraviolet absorption of cholesterol.

A sample of cholesterol, m.p. 148.5°, prepared from brain by Professor Drummond, and a sample obtained from cod-liver oil and recrystallized by them three times, m.p. 148.5°, both showed shallow absorption bands (Af 6) near 2800 and 2900 Å., together with considerable absorption throughout the ultraviolet region (Figure 7*a*). "Examination of the curves," they say, "seemed to indicate to us that the absorption curve was made up of two separate curves, one of which we regard as being due to cholesterol itself and the other to the material which becomes active after absorbing light energy."

To test this point of view they recrystallized the unused cholesterol from ethyl acetate, and examined the absorption of the least soluble fraction (2 g.), which "gave higher extinction, a higher definition for the bands, and made the existence of a third band near 2690 Å. certain" (Figure 7*b*).

Believing these preliminary results to indicate the characteristic absorption to be due to a foreign material in the cholesterol, they undertook more extensive fractional crystallizations. Starting with 200 g. of the crude material, they carried out repeated crystallizations from ethyl acetate. The first crystallization, from 1,500 c.c. of ethyl acetate, yielded 50 g. of crystals, m.p. 147.5—148.0°. This fraction was dissolved in the same volume of ethyl acetate and allowed to crystallize slowly, yielding 25 g., m.p. 148.5°, "the melting point being



a, *b*, *d*, *e*, concentration and solvent not stated, *c*, 2 g in 50 c.c ether, 4 cm. cell, *c'*., "2 g. in alcohol, 4 cm cell", *f*, 0 1 mm. oil; (GG).

Fig. 7—Absorption of Various Cholesterol Preparations [Morton, Heilbron, and Kamm (16; 1927)]

Curve *a*, cholesterols from brain and cod-liver oil; curve *b*, same cholesterol recrystallized from ethyl acetate; curves *c* and *c'*, products of repeated recrystallizations from ethyl acetate; curve *d*, product of a second series of crystallizations ("curve *d* . . . corresponds with greater absorption than curve *c*'"); curve *e*, material from filtrate of 117 g. recrystallization; curve *f*, cod-liver oil; curve *4d*, values of curve *d* multiplied by 4.

if anything slightly higher than that of the pure brain cholesterol specimen obtained from Professor Drummond." The

25 g. of material was recrystallized from 600 c.c. of ethyl acetate, yielding 5 g. This was dissolved in 120 c.c. ethyl acetate, and yielded, after cooling, 2.5 g., m.p. 148.5°.

MARKED INCREASE IN EXTINCTION ON FRACTIONATION

Examination of the final product (soluble to 2 per cent at room temperature as compared with 10 per cent for the original cholesterol) in ether and in alcohol solutions showed that the absorption had increased to approximately four times (that is, extinction coefficients were four times as great) as compared with the "purest material previously obtained," and that the selective absorption bands were much more definite (Figure 7*c* and 7*c'*).

In a new series of recrystallizations, they started with 2,000 g. of the crudest cholesterol and obtained the following successive yields from large volumes of ethyl acetate: 450 g., 117 g., 43 g., 6 g. The final 6 g. was recrystallized from dilute alcohol with a yield of 5 g. The absorption of this material is shown in Figure 7*d*—"the extinction coefficient has again increased beyond the highest point previously recorded, but, on the other hand, the bands, rather contrary to our expectation, have lost somewhat in definition."[1]

The m.p. of this final product was lower and indefinite over the range 146.5 to 149°.[2] This they feel is consistent with the presence of a contaminating compound in cholesterol. The contaminant is contained almost entirely in the least soluble portion, they say, because they obtained from the filtrate from which the 43 g. portion was crystallized a product of considerably higher m.p. (148.3°) which showed but slight selective absorption, and they obtained from the filtrate of the previous (117 g.) crystallization a material of still higher m.p. (148.5) which showed no selective absorption (Figure 7*e*).

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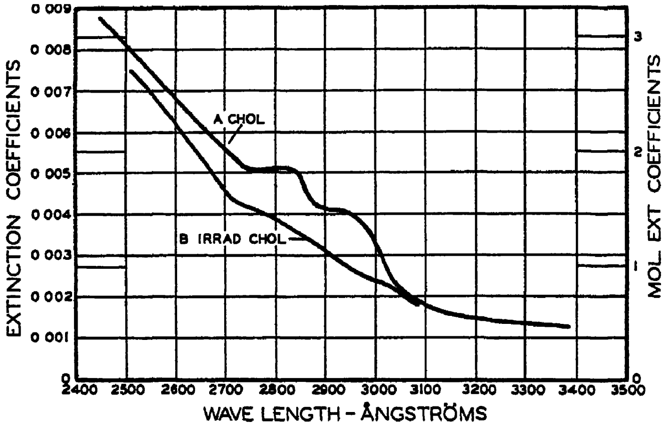
[1] Curves *c* and *c'* in Figure 7 are taken from one figure in the original article, and curve *d* is taken from another figure. Under the latter figure it is said that curve *d* "corresponds with greater absorption" than curve *c'*, although the actual values on the curves indicate that *d* represents only about one-fourth as great absorption as *c'*. One must guess at the reason for the discrepancy, as no explanation is made, and the concentration and cell thickness for curve *d* are not given. If one multiplies the absorption values of curve *d* by four in order to make it compare with curve *c*, one obtains curve *4d*, in which the bands are, if anything, more distinct than those of *c* and *c'*. This result is contradictory to the statement that the bands have "lost somewhat in definition." Two of the bands of curve *d* are shifted toward shorter wave-lengths as compared with *c* and *c'*. No explanation of this is given.

[2] Note that in their previous series of crystallizations the final product had a m.p. of 148.5°.

The addition of sodium ethoxide to an alcoholic solution of Professor Drummond's brain cholesterol increased the definition of the 2690 Å. band "at the expense of other bands," and the addition of HCl caused the appearance of a new band at 3180 Å., which is not far from a second band of cod liver oil recorded by Schlutz and Ziegler (5), "and non-confirmed by ourselves" (Figure 7*f*).



4 g in 250 c c alcohol, 10 cm cell (GG)

Fig. 8—Irradiated and Non-Irradiated Cholesterol
[Morton, Heilbron, and Kamm (16; 1927)]

Irradiation of cholesterol in a solid state between quartz plates, and in alcohol, or ether solution resulted in the disappearance of selective absorption (Figure 8). Irradiation of the fractionated materials likewise resulted in disappearance of the bands.

The authors conclude that there must be a contaminating substance present in cholesterol, identical with provitamin D, to which they give the name "X substance," because: (1) the selective absorption of cholesterol becomes more definite and of a higher extinction coefficient when the material is especially crystallized; (2) it has been shown for cholesterol that the selective absorption bands disappear upon irradiation with ultra-violet coincident with the appearance of vitamin potency.

Heilbron, Kamm, and Morton suggest that the failure of Hess, Weinstock, and Sherman in previous work to obtain activation of cholesterol irradiated in acetone was due to absorption by acetone throughout the region of selective absorption by "cholesterol."

To account for the apparent loss of definition of the selective absorption bands in the end fraction of their last series of recrystallization, they offer two possibilities:

First, "It is quite conceivable that a material very rich in vitamin precursor should undergo photochemical change very readily, even to the point of militating against its detection by spectrographic methods." This argument is evidently based upon the supposition that irradiation of a concentrated solution for a given length of time results in a greater percentage of the original substance being changed than irradiation of a weak solution of the same substance for the same time. The actual truth of the matter is just the reverse, of course, because of the fact that in the more concentrated solution the intensity of radiations penetrating to the farther layers is less.

Second, "Cholesterol itself may consist of a mixture of inactive stereoisomerides (compare Anderson and coworkers (1926) on sitosterol [17]) which might differ in absorption spectra. Stereoisomerides frequently exhibit parallel curves differing only in extinction." It is rather difficult to understand, however, why one stereoisomeride should consistently increase in concentration up to the last fraction, when another should begin to make its appearance pronounced and the former should become less evident.

Assuming that cholesterol has purely general absorption increasing toward the shorter ultra-violet, and that the contaminating "X substance" has approximately symmetrical selective absorption, they append a curve showing geometrically the combined effect of the two (Figure 9). This curve resembles closely those obtained for their fractionated materials, and is used by them to explain the lesser distinctiveness of the 2700 Å. selective absorption band in the fractions they examined.

WHY THE IMPURITY IN CHOLESTEROL ESCAPED DETECTION

A more detailed report of the work of Rosenheim and Webster, with brominated cholesterol, was published in 1927 (18). They suggest that the impurity postulated by them to be present in cholesterol as the precursor of vitamin D had escaped recognition for more than one hundred years in such a well-crystallized substance as cholesterol because it occurs in such minute amounts (1 to 2,000).

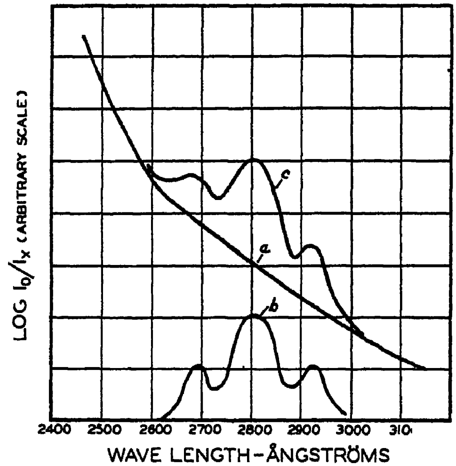


Fig. 9—Effect of Super-Position of General and Selective Absorption [Morton, Heilbron, and Kamm (16; 1927)]

Curve *a*, general absorption; curve *b*, selective absorption; curve *c*, combined effect of the two.

They point out the value of these new discoveries in enabling one to substitute a spectroscopic for a biological test in determining activatability, saying in this regard, "Although such a test had already been tentatively suggested by Schlutz and Morse (3) a firm basis for its application was only established when we showed by animal experiments that the absence of absorption bands in the ultra-violet coincided with the loss by cholesterol of its property of becoming antirachitic on irradiation. It is evident that the destruction or absence of the provitamin can be rapidly proved by negative spectrographic tests, whilst

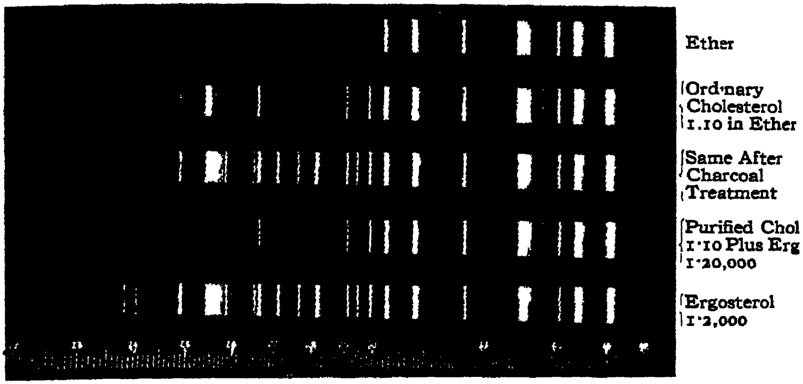


Fig. 10.—Comparative Absorption of Cholesterol and Ergosterol [Rosenheim and Webster (18; 1927)].[1]

animal experiment remains indispensable in confirmation of positive spectrographic evidence."

EXTREME ACTIVITY OF IRRADIATED ERGOSTEROL

Further work on the activity of irradiated ergosterol is reported: "A daily dose of 1 10,000 mg. of irradiated ergosterol cured and prevented rickets in rats kept on a rachitogenic diet. Tests with still smaller quantities, not yet completed, indicate that the limit of activity will prove to be less than 1/50,000 mg. Irradiated ergosterol is therefore the most potent antirachitic substance known, 5 mg. being approximately equivalent to one liter of good cod-liver oil."

They also investigated the antirachitic activity and absorption spectra of stigmasterol and sitosterol prepared from phytosterol by methods involving bromination. These sterols were found to have no antirachitic activity upon irradiation and did not exhibit the characteristic absorption bands of ergosterol or of "ordinary" cholesterol before irradiation.

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[1] They present another plate, showing similar spectrum photographs of ergosterol in various dilutions, which it was not thought worth while to reproduce here.

Cholesterol prepared through the chloride and acetate failed to show the characteristic absorption spectra. Upon irradiation of cholesterol until the vitamin had been formed and destroyed, it was found possible to recover pure cholesterol, from the mixture of cholesterol and provitamin destruction products, by crystallization, the pure cholesterol being free from the characteristic absorption bands.

Charcoal removed the characteristic bands from cholesterol, both Norit and Merck's blood charcoal being employed. They point out that commercial cholesterols vary in their provitamin content, due to the fact that some of them are purified by way of charcoal.

Potassium permanganate in acetone solution removed the characteristic bands.

FORMATION OF A DIGITONIDE BY THE PROVITAMIN

To prove that the provitamin formed a digitonide, they precipitated "ordinary" cholesterol with digitonin, recrystallized the digitonide from pyridine-alcohol, washed the crystals with ether, and dried them. They then fractionally extracted the product with boiling xylene, fraction I representing the cholesterol obtained after the first eight hours of extraction. Examination of fraction I showed increased ultra-violet absorption, equal to about double that of the original cholesterol. Both this fraction and a second one, resulting from a second eight hour period of extraction, proved to be activatable by ultra-violet light as determined by animal experiments.

The spectra were photographed (Aa2) with a 10 mm. quartz cell, using pure ether "free from peroxide" as a solvent. Even in dilutions of 1 to 2,000, ergosterol was found to absorb all the region of the ultra-violet from 3100 Å. towards shorter wavelengths. The "addition of ergosterol 1: 20,000 to a solution of pure cholesterol 1:10"[1] produced an absorption spectrum comparable to that of "ordinary" cholesterol one to ten, from which it was concluded that the absorption of ergosterol is about "2,000 times that of ordinary cholesterol."

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[1] One assumes that this implies the addition of one part of ergosterol to 20,000 parts of the cholesterol solution.

Rosenheim and Webster consider the gradual disappearance of the characteristic band taking place during irradiation to coincide with the formation of vitamin D, which they say is also evidenced by the decrease in digitonin precipitability. By exposing a 1 to 1,000 solution of ergosterol to the action of ultra-violet for various lengths of time, they found that the characteristic bands disappeared completely after about five hours irradiation, and that after this time the product did not have the property of forming an insoluble digitonide.

POSSIBLE MODE OF ORIGIN OF THE PROVITAMIN IN CHOLESTEROL

Jendrassik and Kemenyffi (19; 1927) arrived at the conclusion that formation of the provitamin in hydrated cholesterol proceeds in the natural state until an equilibrium is reached, removal of the provitamin destroying the equilibrium and resulting in further formation until equilibrium is reestablished. They are thus of the opinion that the provitamin is a natural concomitant of hydrated cholesterol, as opposed to the view that it is merely a contaminant thereof.

They base their conclusions on experiments in which successive fractionations of irradiated (active) cholesterol with ethyl alcohol and re-irradiation of the inactive cholesterol residue resulted in the same degree of re-activation, even when the process was repeated several times[1]; and on experiments in which cholesterol brominated, reduced, and treated with water was found to be activatable. The work of Schlutz and Ziegler (5) regarding the absence of selective absorption bands in dehydrated cholesterol and their reappearance upon subsequent hydration is cited as an indication that provitamin formation proceeds only in the hydrated form of cholesterol. Though their finding that irradiated ergosterol is extremely active bears out the conclusions of others that ergosterol is the provitamin, Jendrassik and Kemenyffi do not limit themselves to this viewpoint.[1]

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[1] Actually the filtrate fraction from the first recrystallization gave line tests of +++++, ++++ (8 mg. per day) while that from the third, and last, recrystallization gave ++++, +++ (8 mg. per day). Although the fraction from a single recrystallization gave tests of +++++, +++ in 3 mg. daily doses, and ++++, ++ even in 0.8 mg. doses, the succeeding active fractions were tested only in 8 mg. doses (in all but one instance, two animals per sample). Without the results of graded doses, using more animals per sample, your reviewer sees no convincing argument that the succeeding fractions were equally activatable. The cholesterol was fractionated and the inactive fraction re-irradiated only three successive times. It is quite possible that a considerable proportion of unchanged ergosterol remained after each irradiation, and that this accounts completely for the results, despite the denial of this explanation by the authors on the grounds that "the third inactive fraction . . . did not noticeably lose its activatability." Since this paper is not concerned with absorption spectra, more detailed discussion must be deferred until later.

EXPERIMENTS IN OVER-IRRADIATION OF ERGOSTEROL AND FRACTIONATION WITH ALCOHOL

In a later paper Jendrassik and Kemenyffi (20; 1928) accept ergosterol as the true vitamin precursor, and describe experiments with irradiated ergosterol in which over-irradiation was found to destroy the vitamin potency, fractionation with ethyl alcohol led to a product five times as potent as ordinary irradiated ergosterol, etc.

APPARENT ACTIVATABILITY OF ZYMOSTEROL

In view of the evidence thus far presented, one might assume that the identity of ergosterol as the one true precursor of vitamin D had been established. All workers were not ready as yet to accept ergosterol in this light, however.

Fabre and Simonnet (21; 1929) investigated the ultra-violet absorption (A3) and biological activity of non-irradiated and irradiated zymosterol. Their curves (Figure 11) show only general absorption, without characteristic peaks, and little if any change upon irradiation. The irradiations were carried out at low temperatures in ether solutions for forty-five minutes, in an inert atmosphere. It is pointed out that while ergosterol presents noticeable absorption in alcohol solution 1 to 10,000 or 1 to 100,000 in a 5 mm. cell, zymosterol must be in a concentration 1 to 5,000 to exhibit appreciable absorption under like conditions.

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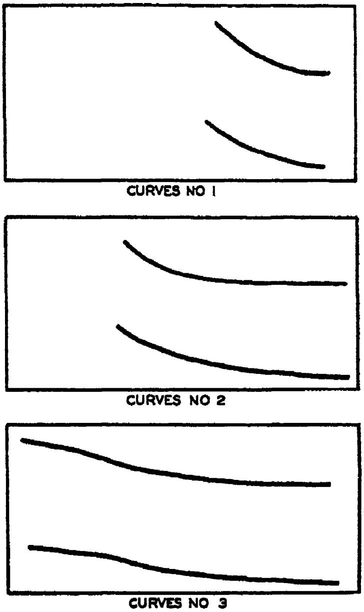
[1] This paper has been widely misinterpreted as presenting the viewpoint that ergosterol is not the precursor of vitamin D, or not the sole precursor [for example, see Sherman and Smith (44; 1931)], despite the fact that it contains such clear statements as: ". . . we do not wish to assert that the provitamin is not ergosterol or a substance similar to ergosterol. On the contrary, our experiments with ergosterol which showed its high activatability suggest that it is. We have only to abandon the theory of an admixed provitamin and accept instead of it the theory of a provitamin formed from cholesterol."

Biological tests of irradiated zymosterol indicated slight antirachitic activity, of the order of 1/100 that of irradiated ergosterol. The possibility that this activity was due to traces of ergosterol not detected spectroscopically is considered by the authors. In view of the failure of others to activate zymosterol (page 145), one is inclined to accept contamination with ergosterol as the explanation of the activatability which they found.



All alcohol solutions 1, 1 to 200, 2 cm cell 2, 1 to 2000 1 cm cell 3, 1 to 5000 5 mm cell no scales on curves.

Fig. 11—Zymosterol Absorption [Fabre and Simonnet (21; 1929)]

The different sets of curves represent different dilutions. In each case, the upper curve represents the irradiated sterol and the lower curve the unirradiated sterol.

Bills, Honeywell, and MacNair (22; 1928) believed it advisable to investigate the provitamin further in view of the fact that Bills (23; 1925) had found that cod-liver oil cholesterol recrystallized and purified with charcoal retained the ability to become antirachitic upon irradiation. They studied various purified cholesterol preparations with biological line tests and with absorption spectra made at the Bureau of Standards.

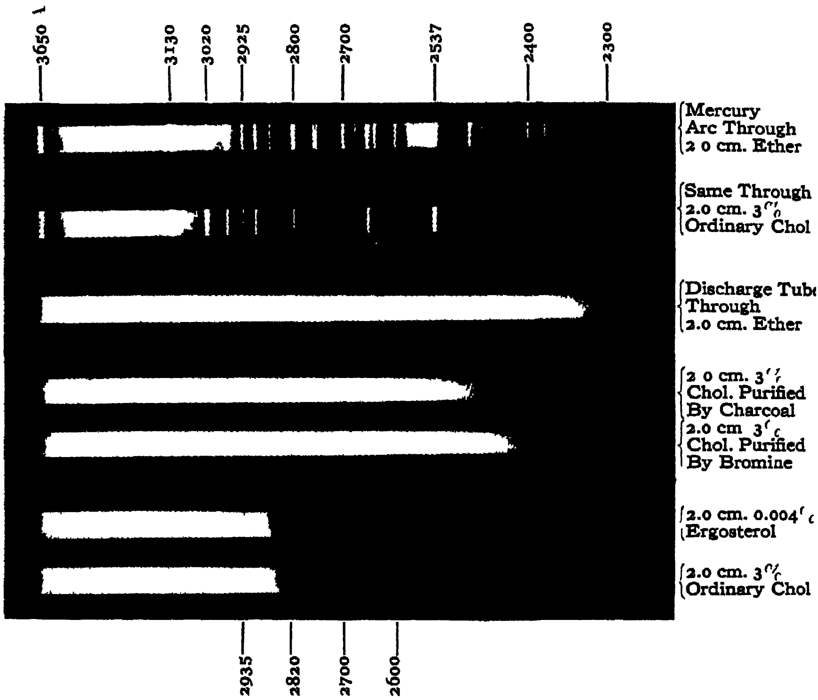


Fig. 12.—Absorption of Cholesterol and Ergosterol [Bills, Honeywell, and MacNair (22; 1928)].

Their cholesterol was prepared from spinal cord, and had a (corrected) melting point of 149°. Animal tests proved it inactive prior to irradiation. Spectrographic tests (Aac2) showed the ergosterol bands at 2935, 2820, and 2700 Å., plainly visible when photographed through a 3 per cent solution, 2 cm. thick. In addition a faint band was visible at 2600 Å. (Figure 12). By making up solutions of various concentrations of ergosterol, photographing the absorption spectra, and comparing the results with the absorption bands obtained with their cholesterol preparations, they came to the conclusion that their cholesterol contained 1.2 ± 0.1 parts per thousand (0.12 per cent) ergosterol.[1] The ergosterol bands were equally apparent whether the ergosterol was put in solution by itself or in a 3 per cent solution of especially purified cholesterol.

IS ERGOSTEROL THE CONTAMINANT OF CHOLESTEROL?

As one way of determining whether or not the contaminant of ordinary cholesterol is ergosterol, they compared the rate of destruction of the characteristic spectra caused by adding $KMnO_4$ to their ordinary preparation of cholesterol, and to cholesterol purified through the bromide, to which had been added sufficient ergosterol to give it practically the same characteristic absorption spectrum. By comparing the final absorption spectrum, after the addition of the reagent, with absorption spectra of solutions having various concentrations of ergosterol, they deduced that about 75 per cent of the contaminant responsible for the selective absorption in ordinary cholesterol was removed by the permanganate, and about 79 per cent of the ergosterol added to the non-absorbing cholesterol was so removed. The close agreement between these two values seemed to them sufficient to establish, from a chemical standpoint, the identity of the contaminant with ergosterol.

Bills, Honeywell, and MacNair point out as a remarkable fact that while ergosterol by itself is described [Tanret (24; 1908)] as being highly unstable, it is sufficiently stable to withstand years of aging when mixed with cholesterol. A sample of gallstone cholesterol kept in the dark, but not out of the presence of air, for sixteen years, was found to have strong characteristic bands of ergosterol. This sample was made highly antirachitic by irradiation. They suggest that cholesterol functions as an anti-oxidant for its admixed ergosterol.

BIOLOGICAL TESTS OF BROMINE-TREATED CHOLESTEROL

Bills, Honeywell, and MacNair examined a number of samples of cholesterol which had been purified with Merck's blood charcoal, or by bromination for varying numbers of times, and also a sample of twice brominated cholesterol obtained from Windaus, of which the absorption had been measured by Pohl and the activity on irradiation had been tested by Hess and found negligible.

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[1] One will recall that Pohl (12) had previously found 0.016% and Rosenheim and Webster (15) 0.05% by the same method.

They point out that previous investigators in testing the biological activity of such materials after irradiation had used only dosages of the order of the mimimum protective dose for ordinary irradiated cholesterol. By using larger doses, they found that in each case about thirty times the dose of their irradiated material would give protection as compared with the required dose of their ordinary irradiated cholesterol. The various irradiated and purified cholesterols were therefore believed to be active in doses thirty times as great as ordinary irradiated cholesterol.

ERGOSTEROL BANDS IN BROMINE-TREATED CHOLESTEROL

Tests of these purified specimens before irradiation in 2 cm. thicknesses of 3 per cent solution having failed to show the characteristic ergosterol absorption bands, but, on the other hand, considerable transmission to 2400 Å., it was decided to photograph the absorption of thicker layers to see if faint ergosterol bands could be detected. A spectrogram taken through a 20 cm. layer of a 15 per cent solution of the most highly brominated sample of cholesterol showed five faint bands, with maxima at 3150, 3040, 2935, 2820, and 2690 Å. The last three of these were believed to be ergosterol bands. This seemed to establish qualitatively the presence of ergosterol in the bromine-treated cholesterol samples, but the authors wished to check this matter quantitatively.

CONCLUSION THAT THE ACTIVABILITY OF CHOLESTEROL CANNOT BE ACCOUNTED FOR BY ERGOSTEROL ALONE

Comparison with ordinary cholesterol showed that a 0.1 per cent solution in the 20 cm. cell showed the ergosterol bands with the same definition as the 15 per cent solution of the repeatedly brominated sample. This indicated to them that if the selective absorption bands were those of ergosterol, as supposed, in the highly brominated sample, then the ergosterol was present in this sample to the extent of about 1/150 that in ordinary cholesterol. Having found the activatability of the brominated sample to be about 1/30 that of ordinary cholesterol, however (five times that consistent with the quantity of ergosterol deduced to be present), they concluded that the observed activatability must be largely due to something other than ergosterol.

One should consider, however, the limits of accuracy of both the spectrographic and biological experiments used as a basis for these deductions. It is hardly possible that the over-all accuracy is such as to make it possible to eliminate 5 to 1 variations.[1]

They then proceeded to question whether even one fifth of the activatability of the highly brominated cholesterol preparation is due to ergosterol. "It is difficult to believe that bromination, which destroys more than 99 per cent of the ergosterol when applied once[1] should fail to destroy the remainder when applied a second time." On the other hand they thought that it was equally improbable that the 2935, 2820, and 2690 bands found in the highly brominated sample were not due to ergosterol. They therefore suggested the bare possibility that traces of ergosterol might be formed continually as degradation products of cholesterol. The two compounds are pointed out to be certainly inseparable in nature.

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[1] The authors publish only the spectrum photographs reproduced herewith, and do not give the details of their animal experiments, so that critical consideration is difficult, but the following possible criticisms might be mentioned: (1) Quantitative deductions from superficial examination of spectrum photographs, without plotting curves of extinction, are decidedly inaccurate. The influence of differences in absorption of substances other than ergosterol present in the samples is difficult to estimate. Note, for example, that the authors obtained the same spectrographic results in examining ergosterol whether they put it into solution alone or dissolved it (in identical concentration) in a 3 per cent solution of thrice bromine-treated cholesterol, despite the fact that such purified cholesterol is indicated by the experiments of others to have appreciable general absorption. (2) Their deduction regarding the comparative ergosterol content of purified and ordinary cholesterol is based on the assumption that Beer's law holds for ergosterol in the presence of cholesterol, which may or may not be true. (3) The materials were all irradiated under the same conditions, in dry films 0.002 g. per sq. cm., exposed 15 m. at 30 cm. from a 220 volt Uviarc operated at 170 v. across the arc. Variations in such irradiation conditions as the absorption of constituents other than ergosterol, differences in absorption of the ergosterol itself due to differences in concentration, differences in intensity, etc., may account for part of the differences in activatability observed. (4) Even with the careful animal technique for which these authors are noted, there are limits to the quantitative accuracy of animal experiments. One wonders, for example, why (as reported in their paper) they found charcoal-purified cholesterol, once, twice, and three times bromine-treated cholesterol, and a sample of cholesterol treated twice with bromine by Windaus, all to have one thirtieth the activatability of their ordinary unpurified cholesterol.

In a note, reference is made to the work of Jendrassik and Kemenyffi (19; 1927), who interpreted the activatability of cholesterol to be due to an equilibrium existing between cholesterol and the provitamin—"a view we feel is open to dispute."

ASSOCIATION OF 3150 AND 3040 Å BANDS WITH ACTIVATABILITY

Since absorption bands were found at 3150 and 3040 Å. in the highly brominated cholesterol—bands which are not found in ergosterol—Bills, Honeywell, and MacNair suggest that the substance responsible for them might be either cholesterol itself or a hitherto undiscovered impurity persisting as well after three purifications with bromine as after one. Following the almost universal tradition that critical wave-lengths for chemical changes are associated with characteristic absorption bands, they say, "It may explain why the isolated lines 3130 and 3030 Å. exhibited activating power in the recent experiments of Hess and Anderson (25; 1927)."

SUGGESTION THAT THE 3150 AND 3040 Å BANDS ARE DUE TO CHOLESTERILENE

Heilbron, Morton, and Sexton (26; 1928) were led by the work of Bills, Honeywell, and MacNair (22) to investigate the presence of a 2600 Å. band in ergosterol and of bands at 3150 and at 3040 Å. associated by Bills and his co-workers with the activatability of cholesterol purified by repeated brominations. Heilbron and his associates confirmed the presence of a band at 2600 Å. in ergosterol, but only with certainty when a continuous light source was used. With regard to the bands at 3150 and 3040 Å., they call attention to their previous report

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[1] This statement is open to question, as one may see from their spectrographic and biological findings regarding repeatedly brominated cholesterol.

(to be reviewed later) of the ultra-violet absorption of cholesterilene. They attribute to this compound bands with maxima at 2940, 3040, and 3210 Å. The first of these, they say, coincides with the 2935 Å. band of ergosterol, while the other two agree closely with the 3040 and 3150 Å bands of Bills and his associates.

Judging from the instability of cholesterol dibromide and the complex nature of its composition, they suggest that the two bands found by Bills and his co-workers in purified cholesterol may be due to traces of cholesterilene formed during the purification process. They state that the amount of this compound necessary to show the absorption found by Bills and his co-workers would be of the order of 0.01 per cent. They say further that this in no way invalidates the results of Bills regarding the activatability of purified cholesterol, but simply separates this property from the absorption found at 3150 and 3040 Å., "for cholesterilene is not rendered active by irradiation."

In undertaking an investigation of the quantitative photochemical activation of sterols, Kon, Daniels, and Steenbock (27; 1928) thought it necessary to investigate first whether or not ergosterol is the true precursor of vitamin D, having on the one hand the results of Windaus, Rosenheim, Hess, and Pohl for the affirmative and those of Bills, Honeywell, and MacNair, and Jendrassik and Kemenyffi[1] for the negative.

They repeated the experiments of Bills, Honeywell, and MacNair, using twice-brominated cholesterol and cholesterol purified with Norit charcoal and with Merck's animal charcoal. Curative and preventive tests were employed with several different groups of rats, and while complete cures were usually not obtained, there was sufficient curative action to confirm the findings of Bills, Honeywell, and MacNair that these purified cholesterols are somewhat antirachitic when irradiated.

TRACES OF ERGOSTEROL IN BROMINE-TREATED CHOLESTEROL

Kon, Daniels, and Steenbock are, however, of the opinion that the protection is due to traces of ergosterol which remain behind after purification, believing that the low value of ergosterol content estimated by Bills and his co-workers from spectrographic data was due to the ergosterol lines being masked by the absorption of decomposition products.

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[1] See footnote page 138.

They agree with Heilbron, Morton, and Sexton (26) that the bands at 3150 and 3040 Å. found by Bills and his co-workers in purified cholesterol are due to cholesterilene formed by oxidation. "We would completely share their opinion inasmuch as spectrograms taken through 20 cm. of a 10 per cent solution of our inactive cholesterol showed such heavy absorption below 2900 Å. that the presence of various oxidation products absorbing ultra-violet but not activatable cannot be questioned."

DRASTICALLY PURIFIED CHOLESTEROL NOT ACTIVATABLE

A more drastically purified cholesterol sample, purified with potassium permanganate, proved to be entirely inactive when irradiated. They conclude that the activatable contaminant of cholesterol is ergosterol.

The remainder of their paper, regarding the quantum efficiency of activation of ergosterol at different wave-lengths, will be discussed in a later section of this review, inasmuch as it is not primarily concerned with absorption spectra.

The same material was published independently by Kon (28; 1928) in a foreign journal.

IS THE PROVITAMIN AN ISOMER OF ERGOSTEROL AND CHOLESTEROL?

Montignie (29; 1929) held the opinion that the provitamin is an isomer of cholesterol and ergosterol coexistent therewith in small quantities. He associated vitamin D with a phosphorescent blackening of photographic plates (8-10 days exposure) obtained with irradiated ergosterol and cholesterol, and because this blackening action was lost after the materials had stood eight days in darkness he says that the activation of ergosterol or cholesterol to vitamin D is a reversible process which ends in a return to the original material. Commercially pure cholesterol and ergosterol, fractionated three times with alcohol, failed to blacken plates after irradiation, from which he concluded that the provitamin had been removed from each.

Various arguments are advanced that the provitamin and vitamin are isomers of ergosterol and cholesterol. Commercially pure ergosterol had a rotation of $[\alpha]_D = -117°$, and the recrystallized sample of $[\alpha]_D = -119°$, which increase in rotation he associates with removal of the provitamin. "There is, therefore, an active isomer which disappears by successive crystallizations. That is alone activated by the ultra-violet rays." One knows, however, that ergosterol re-purified to a rotation of $[\alpha]_D = -132°$ is even more highly activatable than commercially pure samples. Furthermore, the association of vitamin D with the phosphorescent nature of the irradiated materials, on which his arguments are based, is without any definite evidence—in fact, his own results that recrystallized ergosterol fails to give the fluorescence would indicate that the phenomenon is due to an inactivatable impurity in view of the great activatability of purified ergosterol.

FLUORESCENCE DUE TO TRACES OF OXIDATION PRODUCTS

Rosenheim (30; 1927) is of the opinion that the fluorescence of ergosterol and cholesterol is due to traces of oxidation products and it may be that the phosphorescent effects obtained are due to the same or similar compounds. He has obtained preparations of non-fluorescent ergosterol which are just as activatable as fluorescent samples.

Koch, Koch, and Ragins (31; 1929), having found, in agreement with Bills, Honeywell, and MacNair (22), that purified cholesterol is still activatable, and further that it may be made still more activatable[1] by heating under the proper conditions slightly above the melting point, Koch, Koch, and Lemon (32; 1929), turned to a study of absorption spectra to see whether or not this activatability were due to the presence of ergosterol.

Ordinarily, the compounds examined (Ae2) were dissolved in ether and given two minute exposures through 20 mm. of solution.

Cholesterol purified by boiling with alkali and extracted with ether was found to be more transparent to the ultra-violet than the commercial cholesterol from which it was prepared (Figure 13, *c, d, e*). They found the 2930, 2800, 2720, and 2600 Å. ergosterol bands in appropriate ether dilutions of commercial cholesterol (Figure 14).

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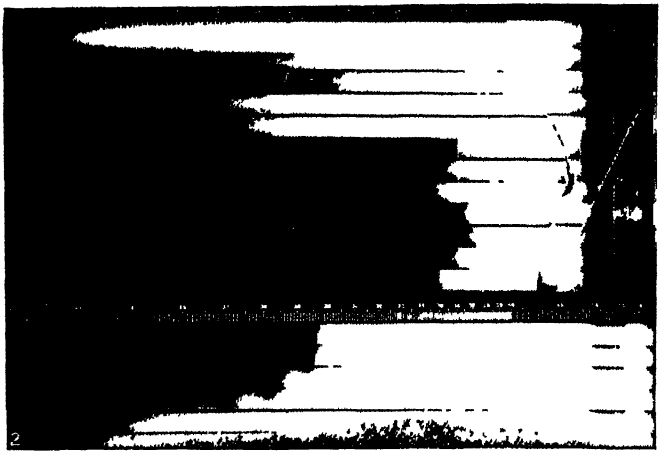
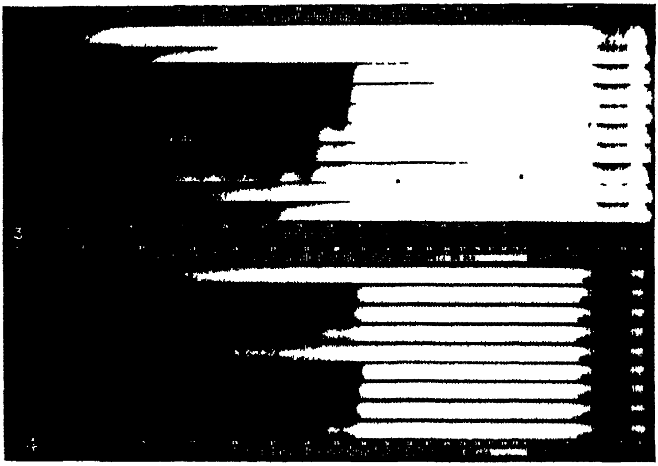
[1] Though never more so than unpurified cholesterol.



A. Cell
B. Solvent
C. 5% Chol. W.
D. 5% Chol. B.
E. 5% Chol A.
F. 5% Chol. H.
G. 5% Chol. II.
H 5% 4 min.
I. 2% Erg. 4 min.
J. 2% Erg. 2 min.
K. 2% 10 mm. 2 min
L. 2% 5 mm. 2 min.
Fig. 13

A. 5% Chol W.
B. 4% Chol. W.
C. 3% Chol. W.
D 2% Chol. W.
E. 1% Chol W.
F. 0.5% Chol. W.
Fig. 14

Exposures for 2 min. through 20 mm cell unless otherwise noted.



A. Cell.
B. Ether.
C. 0.01% Erg.
D. 0.008% Erg.
E. 0.005% Erg.
F. 0.004% Erg.
G. 0.003% Erg.
H. 0.002% Erg.
I. 0.001% Erg.
J. 0.0005% Erg.
Fig. 15

A. Solvent.
B. 5% Chol. E.
C. 3% Chol. E.
D 2% Chol. E.
E 1% Chol E
F. 5% Chol. W.

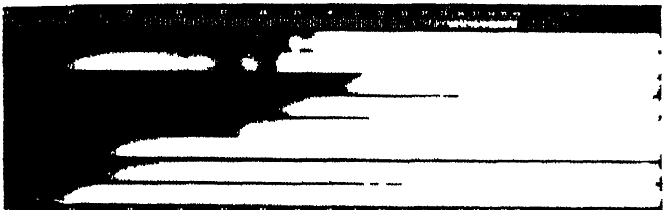
H. 2% Chol. W.
I. 1% Chol. W.
Fig. 16

Exposures for 2 min. through 20 mm. cell.

Figs. 13, 14, 15, and 16—Absorption of Ergosterol and Cholesterol Solutions [Koch, Koch, and Lemon (32; 1929)]

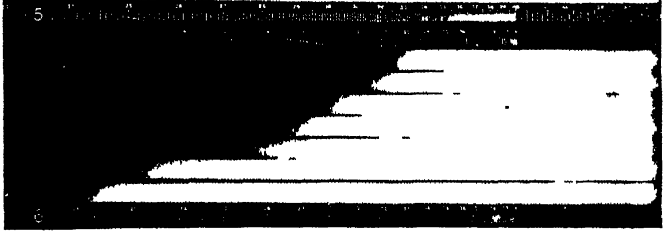
Code: Chol. W—Commercial cholesterol from Wilson Laboratories.
Chol. B—Chol. W purified with NaOH and recrystallized twice from acetone.
Chol. A—Chol B. recrystallized 3 times from acetone.
Chol. H—Unsublimed residue after heating Chol A. 15 hrs. at 225°.
Chol. II—Emulsion layer fraction obtained in washing ether solution of alkali-treated Chol. W. with water.
Chol. E—Cholesterol from chol. dibromide plus 1/1000 part ergosterol.
Erg. I—Ergosterol irradiated in solid form.
Chol. H2—Chol. A. heated, technique not clear from text, but probably 2 hrs. at 165° in nitrogen.
Chol. I—Irradiated Chol. W.
Chol. AI—Irradiated Chol. A.
Chol. II I—Irradiated Chol. II.

(Continued bottom next page)



A 0.004% Erg.
B 0.004% Erg I.
C 2% Chol. II.
D 1% Chol. II.
E 0.5% Chol. II.
F. 0.25% Chol. II
G. 0.12% Chol. II
H. Ether.

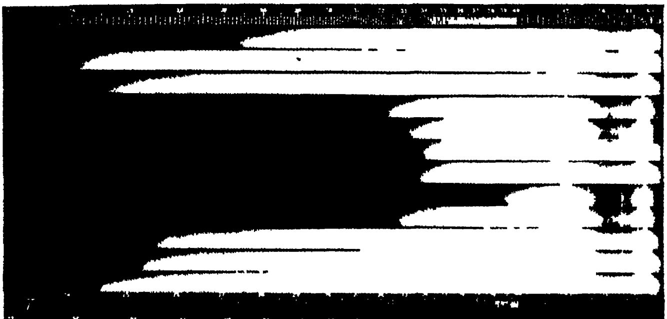
Fig. 17



A 3% Chol. H2.
B 2% Chol. H2.
C 1% Chol. H2.
D. 0.5% Chol. H2
E. 0.3% Chol. H2.
F. 0.1% Chol. H2.
G. Ether.

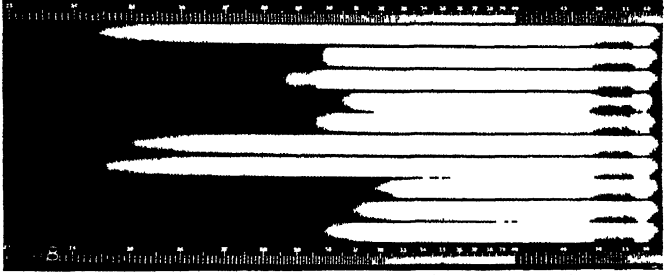
Fig. 18

Exposures for 2 min. through 20 mm. cell



A. 3% Chol. I.
B. 3% Chol. A
C. 3% Chol. AI
D 3% Chol. II.
E 3% Chol. II. I.
F. 3% Chol. H3.
G. 3% Chol. H3I.
H. 3% Chol. H4.
I. 3% Chol. H5.
J. 2% Chol. H6.
K. 2% Chol. H6I
L. Solvent.

Fig. 19.



A. Solvent.
B. 6% Chol. W
C. 6% 10 mm.
D. 3% Chol. D.
E. 3% 10 mm.
F. 6% Chol. Br.
G. 6% 10 mm.
H. 3% Chol. H7.
I. 3% 15 mm.
J. 3% 10 mm.

Fig. 20.

Exposures for 2 min. through 20 mm. cell unless otherwise noted.

Figs. 17, 18, 19, and 20—Absorption of Cholesterol Solutions [Koch, Koch, and Lemon (32; 1929)]

CODE: Chol. H3—Chol. A. heated at 7–10 mm. pressure.
Chol. H3I—Irradiated Chol. H3.
Chol. H4—Chol. A. heated with copper sulphate.
Chol. H5—Chol. A. heated with soda lime.
Chol. H6—Chol. A. heated at 200° for 25 hrs. at high pressure.
Chol. H6I—Irradiated Chol. H6.
Chol. D—Cholesterol Dibromide.
Chol. Br.—Cholesterol from chol. dibromide.
Chol. H7—Cholesterol from dibromide heated 2 hrs. at 165°.

CONFIRMATION OF THE FINDINGS OF BILLS, HONEYWELL, AND MACNAIR

Like Bills, Honeywell, and MacNair (22), they came to the conclusion that there must be some other substance than ergosterol responsible for part of the activatability of cholesterol, because a comparison of the spectrum of the ordinary cholesterol with those of various solutions of ergosterol led them to conclude that it contained about 1/1,000 part ergosterol, while their biological experiments showed it to be about 1/100 as activatable as ergosterol.

Actually there is considerable difference throughout the entire absorbing region, as one can readily see. But granting their statement, one is still not justified in deducing from this comparison that the cholesterol contains only 1/1000 part ergosterol. Allowance should be made for the general absorption of the cholesterol, which Pohl's curves indicate might account for half of the extinction. Further, if instead of comparing Figure 14 *d* with the ergosterol spectra, one compares instead Figure 16 *h* (which is apparently a spectrogram of the same material in the same concentration), one sees that the closest match is obtained with the spectrogram of the 0.008 per cent ergosterol sample. Even without making allowance for the general absorption of the cholesterol, this indicates that the latter is contaminated with 1/250 part ergosterol, instead of only 1/1000 part. Were general absorption properly accounted for, perhaps these spectra would lead one to deduce that the cholesterol is contaminated with ergosterol to the extent of as much as 1 part in 100—the value indicated by the biological experiments.

As further evidence of the inaccuracy of the spectrographic methods involved, one may quote the following statement of the authors relative to Figure 13: "The very striking change in the absorption of purified cholesterol when heated and also the great similarity in the heated cholesterol spectrum and that of Fraction II [the emulsion layer fraction], both highly activable preparations, to that of ergosterol, at least offered some confirmation to our biological reactions, described in the previous paper." The ergosterol spectrum upon which these comparisons are based was made with so concentrated a solution that it absorbed completely all radiations of shorter wave-length than about 3500 or 3600 Å., completely obscuring the entire region in which the characteristic ergosterol absorption occurs.

In view of these vagaries, and the considerations outlined in the footnote at the bottom of page 143, one finds it difficult to accept that a degree of activatability inconsistent with the amount of ergosterol present has been demonstrated.

The ergosterol bands seemed to them to be obscured by a certain amount of general absorption which they correlated with the additional activatable factor. They believed they concentrated this substance in an emulsion layer fraction when purifying cholesterol by boiling with alkali and extracting with ether. This fraction had about the same potency as the original cholesterol when irradiated, but "showed no ergosterol bands"—only general absorption (Figure 17, *c*, *d*, *e*, *f*, *g*).[1]

Samples of cholesterol heated at reduced pressures (7–10 mm.) in an atmosphere of nitrogen showed increases in activatability and in this "general absorption," while samples heated in nitrogen at atmospheric pressure for times up to seventy-two hours at 200–260° C. showed only slight increases in activatability and little if any changes in absorption.[2] Cholesterol similarly heated at very low pressures, of 1 to 2 mm., showed neither changes in absorption nor in activatability. On the other hand, in heating cholesterol with reagents such as P_2O_5 and $CuSO_4$, which destroyed considerable cholesterol, they frequently found strong general absorption without increased activatability (Figures 18, 19, 20).

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[1] Note, however, that even in a 1 per cent solution (Figure 17 *d*) this material absorbed completely throughout the entire region of the ergosterol bands. The general absorption of other materials present may have been so great that the ergosterol bands were masked completely.

[2] It is possible that the general absorption of materials other than ergosterol in their heated samples—an absorption greatest in the short-wave region—acting as a filter, resulted in the irradiation of the ergosterol present under better conditions for activation, so that the irradiated ergosterol in these samples was actually of higher potency than that in the samples of unheated irradiated cholesterol. If this were true, the increased activatability would not be the result of an increased provitamin content, but merely of an alteration of the conditions of irradiation. The samples were irradiated dry, in nitrogen, at 40 cm. from a Cooper Hewitt lamp, for 35 min.

Following the statements of previous workers that cholesterol purified by bromination while boiling with potassium permanganate in acetone lost its activatability, they tested the activatability of samples of cholesterol purified by the bromine treatment and by a triple treatment with potassium permanganate in boiling acetone. Both samples when irradiated were found to be active in doses of thirty to seventy times that of ordinary irradiated cholesterol. These findings, they believe, bear out the conclusions of Bills, Honeywell, and MacNair. The purified samples in both cases showed increased activatability when heated under appropriate conditions.

INACTIVATABILITY OF DIGITALIGENIN

Pursuing the question of whether or not ergosterol is the only parent substance of vitamin D, Rosenheim and Webster (33; 1928) studied the ultra-violet absorption and activatability of digitaligenin. They first discuss the apparent necessity of three double bonds for activation, as found by various workers. They then consider the possibility that various iso-ergosterols might be activated.

They irradiated a sample of Reindel's iso-ergosterol (Reindel, Walter, and Rauch, 34; 1927) from Windaus, and tested it for antirachitic potency, finding it inactive both before and after irradiation. Neo-ergosterol, they say, has been found to be inactive after irradiation (Windaus and Borgeaud, 35; 1928). Zymosterol, the only other naturally occurring sterol of this type, they would not expect to be activatable on account of the fact that it has no selective absorption in the ultra-violet. In a note they state that Hume, Smith, and Smedley-MacLean (36; 1928) have established that irradiated zymosterol has no antirachitic properties.

They state that the activatability of ergosterol would be unique were it not for the statement of Windaus (37; 1927) and Windaus and Holtz (38; 1927) that digitaligenin $C_{23}H_{30}O_3$, structurally related to cholic acid, can be activated by irradiation. This substance contains three double bonds. They examined the activity of this substance after irradiation of from thirty minutes to two hours in 0.01 and 0.2 per cent alcoholic solution, with negative results.

SPECTRUM OF DIGITALIGENIN

Rosenheim and Webster believe these results to be in harmony with their spectrographic findings, which showed intense absorption in the ultra-violet, quite different however from ergosterol. The substance showed one band, with a maximum at 3400 Å., extending from 2700 to 3900 Å., with possibly secondary bands in the region below 2500 Å. The broad absorption band stayed practically unchanged after two hours irradiation, "whereas the absorption spectrum of ergosterol rapidly changes under these conditions."

They believe the results of Windaus and Holtz (38) were due to the fact that their samples of the substance were irradiated in a 0.01 per cent solution in olive oil, the olive oil being activated. Believing that the irradiated olive oil gave protection in Windaus's work, they feel that the evidence is strengthened for the assumption that ergosterol is the specific parent substance of vitamin D. The control experiment of Windaus and Holtz in which olive oil was irradiated for three hours with the magnesium spark is criticized on the basis that the high absorption of the digitaligenin was lacking, and the time of irradiation was prolonged so as probably to destroy any vitamin D potency which might be built up during the course of irradiation.

ACTIVATABILITY OF STEROLS OF ERGOT

Rosenheim and Webster made an attempt to determine whether or not the second mycosterol occurring in ergot, given the name fungisterol by Tanret, could be activated (39; 1927), but this early attempt led to indecisive results. They repeated the experiment later (40; 1929). Parallel biological and spectrographic examination of this sterol led them to the conclusion that such activatability as is found is due to contamination with up to 5 per cent of ergosterol.

A second specimen of apparently the same sterol prepared from ergot by another pharmaceutical house did not agree in physical constants with the first sterol, but neither agreed with the fungisterol described by Tanret. They concluded that Tanret's sterol was actually a mixture of several sterols, and they say that two further constituents of this mixture were isolated and investigated at the Burroughs Welcome & Co. Laboratories.

Compared spectroscopically with solutions of ergosterol, sterol I showed no indications of ergosterol being present. It gave a negative antirachitic test. Sterol II showed about 8 per cent ergosterol present, and the same order of activity upon irradiation as the irradiated fungisterol sample previously described.

These results, they believe, further bear out the conclusion of Rosenheim and Webster (33) that ergosterol is the specific parent substance of vitamin D.

Hess and Anderson (41; 1927) found that sitosterol prepared from corn oil could be activated by ultra-violet irradiation, but that it could not be activated after purification by bromine treatment.

SUMMARY

Summing up, the unanimous opinion now is that provitamin D is not cholesterol but a contaminant or transformation product thereof. The majority hold that ergosterol is the one true provitamin. The dissenters from this viewpoint, Bills, Honeywell, and MacNair (22), Koch, Koch, and Ragins (31), Koch, Koch, and Lemon (32) on the one hand have based their conclusions mainly upon inaccurate absorption data, the quantitative applicability of which is questionable; and Montignie (29) on the other hand on the unsupported association of vitamin D with phosphorescence. It seems probable to the reviewer that the activatability of zymosterol found by Fabre and Simonnet (21) is explained by contamination with ergosterol, since others have found it definitely inactivatable.

One must, therefore, consider the opinions of the dissenters with reservations and incline toward the view that only ergosterol or derivatives thereof are capable of antirachitic activation, at least until further evidence is presented. The activatability of various isomers and derivatives of ergosterol is another question, and one which it seems best to consider in a later section concerning the chemistry of ergosterol and vitamin D as deduced from absorption spectra.

NOTE: The next section will be a continuation of Part I, dealing with the ultra-violet absorption spectra of oils.

A few papers not dealing with absorption spectra have been reviewed briefly in this section because they were considered essential to the argument, but for the most part consideration of such papers has been deferred until later.

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A NOTE ON THE X-RAY ABSORPTION OF ERGOSTEROL

By ELIZABETH SHELOW AND JOHN R. LOOFBOUROW

Recently the authors published a paper regarding the antirachitic activation of ergosterol by soft X-rays (1) in which there appeared a curve of the soft X-ray absorption of ergosterol. After this paper had gone to press, it was discovered that in redrawing the curve for reproduction it had been incorrectly plotted. While its purpose was merely to illustrate the enormous increase in absorption toward longer wave lengths, and while consequently the absolute values of absorption were not essential to the argument, it was thought advisable to present a corrected curve (Figure 1).

Curve *A* shows the K level X-ray absorption of ergosterol calculated from the values of Olson, Dershem, and Storch (2) for carbon, hydrogen, and oxygen (Table 1) on the basis of the empirical formula $C_{27}H_{42}O$ for ergosterol, and on the assumption that the cube law:

$$\left(\frac{\mu}{\rho}\right)_K = C\lambda^3 + \frac{\sigma}{\rho} \qquad \left(\frac{\tau}{\rho}\right)_K = C\lambda^3$$

holds for these elements throughout the region in question.

More recent measurements by Allen (3) give higher values for the absorption of the elements concerned (Table 1). Using these values, and the cube law, one obtains curve *B*.

It is well known that X-ray absorption departs considerably from the λ^3 law in the region of long wave-lengths (from, say, 0.5 Å.) considered here. Allen (4), on the basis of measurements of many elements to 4.0 Å., came to the conclusion that a fairly close approximation to the actual absorption may be calculated by using $\lambda^{2.92}$ instead of λ^3. Curve *C* was calculated in this way, using Allen's values for the elements concerned.

More recently Allen (5) has found that the wave-length exponent is probably an exponential function of the wave-

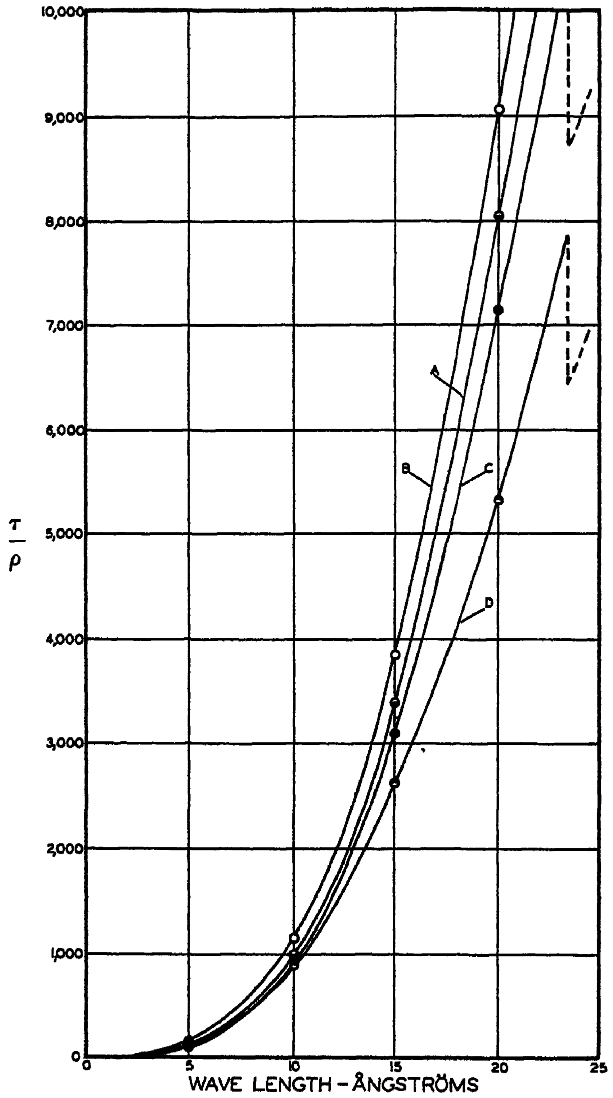

Fig. 1—X-Ray Absorption of Ergosterol

Curve *A*, $\frac{\tau}{\rho} = C\lambda^3$, using values of Olsen, Dershem, and Storch for calculating C; curve *B*, same, using Allen's values for C; curve *C*, Allen's values, $\frac{\tau}{\rho} = C\lambda^{2.92}$: curve *D*, Allen's values, $\frac{\tau}{\rho} = C\lambda^{(2 + \epsilon^{-00.1\lambda})}$

length, the following expression representing a close approximation:

$$\left(\frac{\tau}{\rho}\right)_\lambda = C\lambda^{(2+\epsilon^{-a\lambda})}$$

The coefficient a is different for different elements, being about 0.01 for those considered here. The absorption of ergosterol according to this expression, using Allen's values, is shown in Curve *D*.

TABLE I

Fluorescent Absorption Coefficients "C" (Where $\frac{\tau}{\rho} = C\lambda^3$)

ELEMENT	O., D., AND S.	ALLEN
Carbon . .	1.01	1.16
Hydrogen	0.28	0.25
Oxygen .	2.785	2.94

Dr. Allen has kindly measured for us the absorption of a sample of ergosterol ($[\alpha]_{\mathrm{D}}^{0} = -131.7$) at $\lambda = 0.71$ Å., finding $\frac{\mu}{\rho} = 1.02$.

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A PRELIMINARY INVESTIGATION OF THE GROWTH-PROMOTING EFFECT OF SELECTED ULTRA-VIOLET RADIATIONS ON PLANTS

BY ROBERT B. WITHROW AND HARRIS MILLER BENEDICT

SUMMARY

1. A preliminary investigation of the effect of the 2900-3100 Å. region of the ultra-violet spectrum on coleus and tomato plants indicates that this region promotes their growth rate.

2. Small amounts of energy in the ultra-violet of wave-length shorter than 2900 Å. are shown to counteract the stimulatory effect of relatively large amounts of energy in the 2900-3100 Å. region. Consequently, the use of filters which transmit slight amounts of energy shorter than 2900 Å. would be expected to give negative results in botanical investigations of the alleged growth-promoting effect of the region from 2900 to 3100 Å.

INTRODUCTION

Knowing the importance of the region of the ultra-violet near the limit of the sun's spectrum (that is, from about 3100 to 2900 Å.) for animal life, one might consider the possibility that this same region is essential for optimum conditions of plant growth. Various experimenters have, however, come to the conclusion that the exclusion of this region of the ultra-violet has little, if any, effect on plants.

Considering these experiments critically, one may question whether or not the conditions have been so controlled as to obtain sufficient energy in the 2900-3100 Å. region in the case of experiments carried out with sunlight, and whether the shorter wave ultra-violet, known to be lethal for plant life, has been excluded with sufficient thoroughness in experiments carried out with artificial sources.

In view of the early work of this laboratory (1, 2, 3), which indicates that the biological effects of radiations begin abruptly at critical wave lengths in the spectrum and continue for all shorter wave lengths (and that, in particular, lethal effects for both plant and animal life begin at about 2900 Å.) one would expect it to be necessary to exclude very completely all energy of wave length shorter than 2900 Å. when employing artificial sources, in order not to mask possible beneficial effects of longer waves by the detrimental effects of the lethal region.

With these considerations in mind, the following preliminary investigation was undertaken.

REVIEW OF THE LITERATURE

Investigations of the effects upon plants of ultra-violet radiations from 3100-2900 Å., near the limit of the sun's spectrum, fall into two general classes, according to the sources of radiations used. Popp (4), Pfeiffer (5), Shirley (6), and Arthur (7), of the Boyce Thompson Institute, McCrea (8), and others have made use of greenhouses equipped both with ordinary glass and special glasses fairly transparent to this region of the ultra-violet. Other investigators, as Popp and Brown (9), Newell and Arthur (10), Fuller (11), Eltinge (12), and Sheard and Higgins (13), have grown their plants under the usual hothouse conditions. The 3100-2900 Å. region of the ultra-violet not being transmitted by the window glass, they have used artificial sources to supply it, these being filtered with glasses intended to absorb the shorter wave ultra-violet region beyond the limit of the sun's spectrum.

Let us consider first the question of energy in connection with experiments of the first type. The National Carbon Co., Kimball (14), and Fabry and Buisson (15), who made radiometric determinations of the energy in the short ultra-violet region of the sun's spectrum, have shown that the amount of this energy reaching the surface of the earth is decidedly low. Coblentz and Stair (16) have investigated the ultra-violet transmission of various glasses which have been used as window materials in the greenhouses in the first class of experiments. Their results show that new glasses of the most transparent type are capable of transmitting only about 55 per cent of the energy in the 3100-2900 Å. region. These same glasses acquire a film of dust and dirt after a few months' use which may, in extreme cases, reduce the transmission to one or two per cent. Thus, with only a small quantity of the ultra-violet reaching the earth and only a small percentage of it transmitted by the glasses, plants grown in especially glassed greenhouses may be expected to receive very little ultra-violet shorter than 3100 Å, even during the summer months. It seems reasonable to presume that if the 2900-3100 Å. region should have any marked influence on the growth of plants, such effects would not be evidenced by experiments of the first class.

Now let us consider the possible effect of lethal radiations in experiments of the second class. These experiments have been carried on with such artificial ultra-violet sources as carbon arcs or quartz mercury arcs. These sources emit not only the ultra-violet of the sun's spectrum, but also considerable energy of wave length shorter than 2900 Å., which has been shown to have a destructive action upon plant tissues even in very low intensities.

If filters which transmit even a small amount of energy shorter than 2900 Å. are used with such artificial sources, it is possible that any stimulative effect of the 2900-3100 Å. region might be counteracted by the destructive action of the ultra-violet of shorter wave length than 2900 Å.

Most of the previous experiments of the second class have been performed with glass filters. Such glasses are poor filters. They transmit appreciable quantities of the short-wave ultra-violet, as may readily be shown by prolonged exposures with a spectrograph. The conflicting results of previous researches of the second class on the effect of the 2900-3100 Å. region on plant life may be due to the various degrees of transmission of the shorter ultra-violet by the glass filters used in these experiments.

EXPERIMENTAL

ULTRA-VIOLET FILTERS

Ultra-violet filters with sharp absorption limits were developed especially for use in these experiments. They are described in a separate paper (20).

These filters have sharp absorption bands, with little transmission of energy beyond the "limit" of the absorption band. The filtering is so complete that a five-minute exposure through a quartz spectrograph to a mercury arc shows the same mercury lines on the photographic plate as a five-second exposure.

GROWTH COMPARTMENTS

Coleus and tomato plants were used. They were grown in six-inch pots in compartments three feet by six feet, by forty inches high, walled on three sides. The compartments were separated from each other by double partitions of wall board, and black cloths were hung across the fronts to exclude light from sources other than the controlled artificial radiations. Control plants were grown on a window sill with a southern exposure.

TEMPERATURE

The room temperature could not be kept constant throughout the duration of the experiment, but the temperature of the different compartments was watched carefully and kept as uniform as possible.

HUMIDITY

It was not possible to keep the humidity constant, but to maintain it as high as possible, pans which completely covered the base of each compartment were kept half full of water. Two similar pans of water were kept in front of the radiators in the room.

LIGHT SOURCES

Two types of light sources were used for each compartment—tungsten lamps and 50 inch, A. C., Corex, low-pressure mercury arcs.[1] The tungsten lamps supplied, for the most part, visible radiations; and the mercury arcs, both visible and ultra-violet.

To give uniform intensity and to reduce the effects of shading, five 200 watt tungsten lamps were suspended forty inches above the base of each compartment, near one end in order to allow room for the mercury arc. Most of the infra-red rays longer than 14,000 Å., produced by the tungsten lamps, were filtered out by water cells containing two inches of water.

The mercury arcs were hung between adjacent compartments in such a way that half of an arc extended into each compartment. These Corex mercury arcs emit radiations approximating those obtained by using a Corex filter in combination with a quartz mercury arc; namely, considerable intensity in the ultra-violet longer than about 2890 Å., with progressively decreasing intensity of shorter wave lengths to a limit of about 2650 Å.

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[1] These lamps were kindly supplied by the General Electric Vapor Lamp Company.

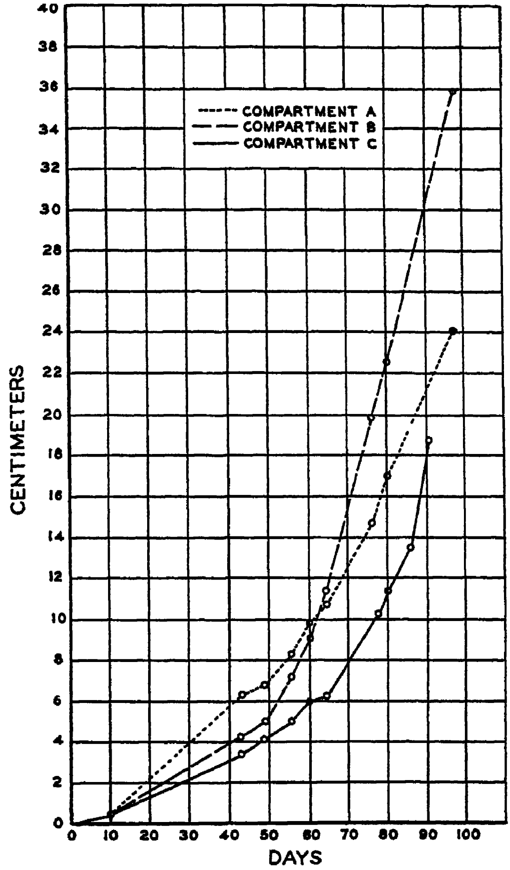

Fig. 1—Average Growth Rates of Coleus

Compartment A, radiations longer than 3130 Å.; compartment B, radiations longer than 2970 Å , compartment C, radiations longer than 2650 Å.

SPECTRAL REGIONS EMPLOYED

The cellophane light filters were suspended on wooden frames below the mercury arcs. Two sheets of cellophane impregnated with potassium hydrogen phthalate were used in compartment A. This filter absorbs practically all energy of wave length shorter than the 3130 Å. mercury line. Two sheets of cellophane impregnated with sodium benzoate were used in compartment B, the limit of transmission being the 2970 Å. mercury line. Three sheets of untreated cellophane were used in place of the filters, in compartment C, transmitting the full

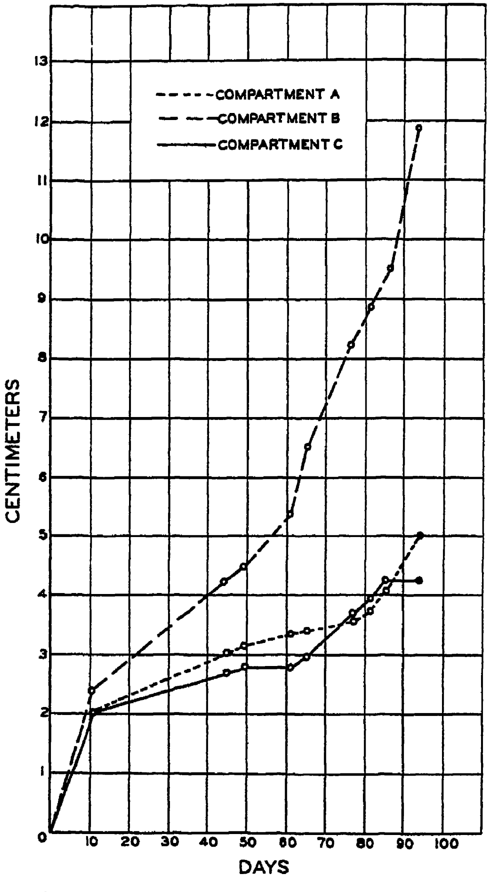

Fig. 2—Average Growth Rates of Tomato

Compartment A, radiations longer than 3130 Å; compartment B, radiations longer than 2970 Å; compartment C, radiations longer than 2650 Å.

spectrum of the mercury arc with the intensity decreased somewhat through absorption by the cellophane. Spectrograms of the radiations in C showed little energy of wave length shorter than 2800 Å., faint lines appearing on the plates to 2650 Å. with prolonged exposures. Compartment C represents the light conditions present in the second type of experiments mentioned in the introduction, in which an artificial source of light, filtered with an ultra-violet transmitting glass, is used.

INTENSITY

The intensity of radiation differed but little in the three compartments, because the ultra-violet region removed by the filters represented but a small portion of the total radiation. The total ultra-violet energy in B, however, was less than that in A, because of the filtering out of the short-wave radiations. Filters for A and B were chosen with a view to equalizing the ultra-violet energy in these two compartments as much as possible, the A filter having a longer-wave absorption limit but greater transmission near this limit.

Measurements made in a later series of experiments with the same illumination equipment, using a Macbeth illuminometer, showed the intensity of visible light at the pots to be of the order of 30 foot-candles. This low intensity resulted in etiolation of the tomato plants.

SOIL AND METHOD OF PLANTING

The soil was prepared by mixing garden loam with well-rotted manure. The mixture was sifted and placed in six-inch pots, all pots being prepared at the same time to insure uniformity. Five pots of tomato and five of coleus were put in each compartment. Eight tomato seeds were planted in each pot, and the seedlings were thinned out to leave four per pot. No definite number of coleus seeds was planted per pot, and the seedlings were not thinned.

DURATION OF EXPERIMENT

The seeds were planted January 7, 1930 and the pots were immediately placed under irradiation. The experiment was concluded April 8, 1930. During this time the plants were irradiated daily from 8 A. M. to 8 P. M., the pots being moved in rotation every day so that each pot occupied each position in its compartment.

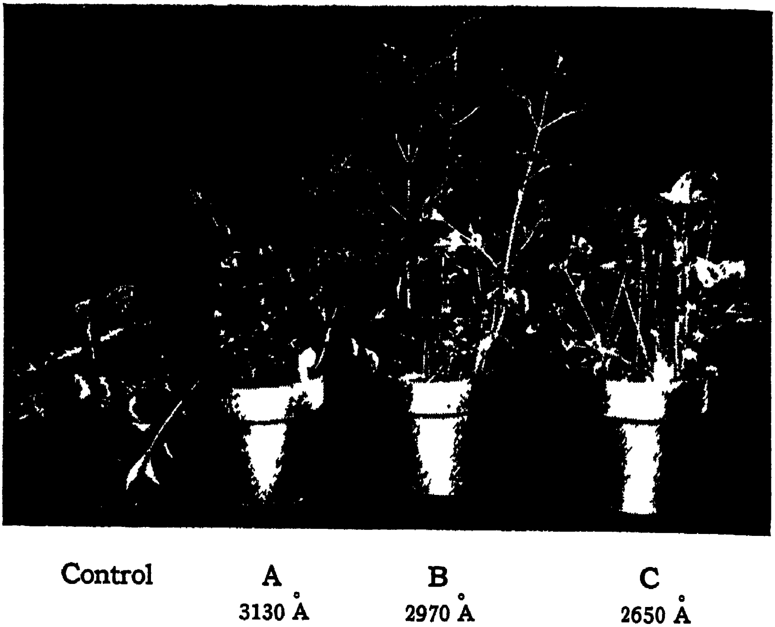

Fig. 3—Typical Pots of Coleus from the Various Compartments.

These pots were not thinned (see text), hence there are more plants in some than in others.

RESULTS

The heights and growth rates of the plants, the lengths of internodes, the diameters of the stems, the areas of the leaves, the number of leaves, and the fresh and dry weights were observed in this experiment.

In compartment C (2650 Å. limit) there were six tomato plants alive at the close of the experiment. In compartment B (2970 Å. limit) there were fourteen, and in compartment A (3130 Å limit) there were only two. All of these plants were measured.

TABLE I

Average Height of Plants in Each Pot, and Averages for Groups of Pots at End of Experiment

COMPARTMENT	SHORT WAVE-LENGTH LIMIT	POT 1	POT 2	POT 3	POT 4	POT 5	AVERAGE 5 POTS
				COLEUS			
A	3130 Å.	20.8	25.3	21.0	21.5	28.6	23.4 cm.
B	2970 Å.	36.0	31.3	33.0	39.0	26.3	33.1 cm.
C	2650 Å.	12.5	18.0	16.5	21.2	19.2	17.5 cm.
				TOMATO			
A	3130 Å.	....	....	4.1	....	....	4.1 cm.
B	2970 Å.	13.6	7.4	7.2	6.8	6.0	8.2 cm.
C	2650 Å.	4.0	3.5	3.5	3.5	....	3.8 cm.

In measuring Coleus plants the three most representative ones in each pot were used. In all the pots, some of the plants shaded the others. For this reason, the plants which received direct radiation only were chosen for measurement.

HEIGHT AND GROWTH RATE

The plants in compartment B (2970 Å. limit) were, in both cases, roughly twice as tall as the plants in either compartment C (2650 Å. limit) or A (3130 Å limit).

The growth rates of the plants in the different compartments are shown in Figures 1 and 2. The age of the plants, from the time of planting the seeds, is plotted against the height in centimeters. In the case of tomatoes, all plants seemed to have about the same rate of increase in height for two weeks, after which the plants of compartment B continued to increase in height at the same rate, while those in compartments C and A slowed down to such an extent that they grew only one to two centimeters during the remainder of the experiment. The coleus plants in all the compartments showed a comparatively regular increase in height, the plants in compartment B outdistancing the others after lagging at the start.

TABLE II

Average Length of Internodes in Each Pot, and Averages for Groups of Pots, at End of Experiment

COMPARTMENT	SHORT WAVE-LENGTH LIMIT	POT 1	POT 2	POT 3	POT 4	POT 5	AVERAGE 5 POTS
			COLEUS				
A	3130 Å.	3.7	4.2	3.7	3.4	4.5	3.9 cm.
B	2970 Å.	4.6	5.4	5.3	5.0	4.0	4.9 cm.
C	2650 Å.	2.4	3.3	3.7	3.5	3.8	3.3 cm.

INTERNODAL LENGTH

The tomato and coleus plants in compartment B had the longest average internodes. All of the internodes on each plant were measured and averaged. These results, measured in centimeters, are given in Table II.

DIAMETERS OF STEMS

Stem diameters were obtained by measuring, in millimeters, the diameter of the stem at the midpoint of all the internodes, and dividing by the number of internodes to obtain the average stem diameter in each pot. The average diameters of group B were greatest, those of group A and C being about equal (Table III).

NUMBER AND AVERAGE AREA OF LEAVES

The average leaf areas were roughly fifty per cent greater in B than in A or C, as shown in Table IV. The number of leaves

TABLE III

Average Stem Diameters in Each Pot and Averages for Groups of Pots, at End of Experiment

COMPARTMENT	SHORT WAVE-LENGTH LIMIT	POT 1	POT 2	POT 3	POT 4	POT 5	AVERAGE 5 POTS
COLEUS							
A	3130 Å.	2.2	2.7	2.1	2.3	2.6	2.4
B	2970 Å.	4.0	3.5	4.3	4.1	3.0	3.8
C	2650 Å.	1.6	2.5	2.7	2.9	1.9	2.3

produced per plant differed only slightly in the three compartments as shown in Table V, but was greater in both instances in compartment B.

FRESH AND DRY WEIGHTS

The fresh and dry weights are given in Table VI. The weights are for the whole plant, root and shoot. The plants of compartment B had both the highest fresh and dry weights, ranging from three to four times as great as those of compartments A and C.

TABLE IV

Average Leaf Areas in Each Pot, and Averages for Groups of Pots, at End of Experiment

COMPARTMENT	SHORT WAVE-LENGTH LIMIT	POT 1	POT 2	POT 3	POT 4	POT 5	AVERAGE 5 POTS
COLEUS							
A	3130 Å.	9.7	11.1	11.1	10.7	12.8	11.1 sq.cm.
B	2970 Å.	21.6	15.0	30.0	16.7	27.4	22.1 sq.cm.
C	2650 Å.	3.1	9.5	9.0	13.4	6.0	7.8 sq.cm.

DISCUSSION

The results of this investigation indicate that the region of the ultra-violet near the limit of the sun's spectrum, which is absorbed by ordinary greenhouse glass (that is, the region from 2900 to 3100 Å.) causes increased plant growth. Absence of all wave lengths shorter than 3100 Å. in compartment A resulted in a general restriction of growth. The plants were not so tall, the internodes were shorter, the stem diameters were less, the leaf areas were reduced, and the fresh and dry weights were much less than in the case of those grown in the presence of ultra-violet radiations of wave-length longer than 2900 Å. (Compartment B). The inclusion of still shorter wave-lengths, between 2900 and 2650 Å. (compartment C), resulted in retardation of growth.

TABLE V

Average Number of Leaves per Plant in Each Pot, and Averages for Groups of Pots, at End of Experiment

Compartment	Short Wave-Length Limit	Pot 1	Pot 2	Pot 3	Pot 4	Pot 5	Average 5 Pots
COLEUS							
A	3130 Å.	12	13	11	13	13	12.4
B	2970 Å.	15	11	13	15	13	13 4
C	2650 Å.	10	11	9	13	10	10 6

As previously mentioned, the intensity in compartment C was slightly greater than the intensities in compartments B and A, which were approximately equal. If the difference in intensity of radiations caused the differences in the plants of the three compartments, the plants of compartments B and A should have been more or less similar, while those of compartment C should have been decidedly different from those of either B or A. Actually, the plants in compartment B showed the greatest deviation, while those of compartments C and A were very much alike. Thus, differences in intensity cannot account for the changes noted.

C. Timiriazev (17) has claimed that, under equal intensities, photosynthesis is carried out at a much greater rate in the red end of the spectrum than the blue-violet end. As the red end of the spectra of the lights of the several compartments was the same, the different results can not be attributed to differences in this region.

TABLE VI

Average Fresh and Dry Weights in Each Group of Pots at End of Experiment

COMPARTMENT	SHORT WAVE-LENGTH LIMIT	NUMBER OF PLANTS	AVERAGE FRESH WT.	AVERAGE DRY WT.	% WATER
		COLEUS			
A	3130 Å.	15	4 04 gr.	.163 gr.	91
B	2970 Å.	14	11 2 gr.	.450 gr.	96
C	2650 Å.	15	2 91 gr.	109 gr.	91
		TOMATO			
A	3130 Å.	2	240 gr	015 gr.	94
B	2970 Å.	14	884 gr.	052 gr.	94
C	2650 Å.	6	30 gr.	016 gr.	95

It is indicated by the above results that the removal of the 2900-3100 Å. ultra-violet region is detrimental to the growth of tomato and coleus plants, and that the inclusion of a small amount of lethal radiation of shorter wave length than 2900 Å. is sufficient to mask the beneficial effect of the 2900-3100 Å. region. It is also evident why experiments with glass filters might lead to negative results for growth promotion by the 2900-3100 Å. region, the radiations in C (where growth promotion was not noted) being, as previously pointed out, equivalent to the rays from a quartz mercury arc filtered by Corex D glass.

Because of the limited number of plants used and the inadequate growth conditions, especially with regard to intensity of illumination, these results are offered simply as preliminary data indicating the possible growth-promoting action of the 2900-3100 Å. ultra-violet region.

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THE NEAR INFRA-RED ABSORPTION SPECTRA OF ERGOSTEROL AND IRRADIATED ERGOSTEROL

BY ELIZABETH SHELOW

SUMMARY

A preliminary investigation of the infra-red spectra between 0.8μ and 3μ of ergosterol and irradiated ergosterol indicates that the alcohol group does not disappear and that the ketone group does not develop as a result of irradiation with ultra-violet light of wave-length longer than 2960 Å.

There is some indication of the presence of ketone derivatives among the products of irradiation with unfiltered ultra-violet light.

The general form of the infra-red absorption curve of ergosterol irradiated with long wave-lengths resembles that of ergosterol and isoergosterol.

These findings suggest that vitamin D is an alcohol isomeric with ergosterol.

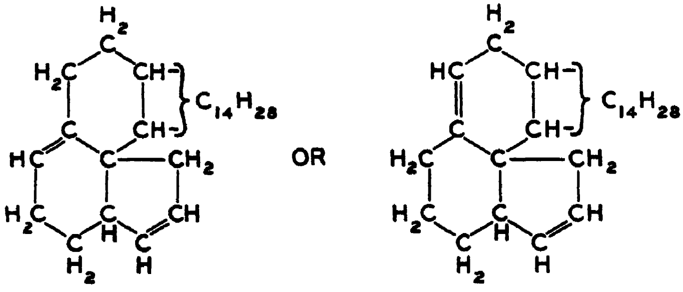
INTRODUCTION

The physiological importance of the antiricketic vitamin has led to a tremendous amount of work on the structure of the parent substance, ergosterol, and upon the nature of the change of ergosterol to vitamin D. Because the infra-red spectrum of a compound depends upon the chemical linkages of the molecule, a study of the infra-red spectra of ergosterol and of irradiated ergosterol offers a promising method of attacking the problem of the nature of vitamin D.

Ergosterol is generally accepted as the parent substance of vitamin D. Since there is some evidence that activatability may be a property of compounds with a certain molecular configuration rather than of one particular substance, there is a possibility that other substances with this configuration may be found. It is certain, however, that vitamin D has the typical sterol grouping and that it develops either from ergosterol or from some substance which shows the same ultra-violet absorption spectrum. Most of the evidence favors the view that ergosterol is the sole precursor of vitamin D.

The structure of ergosterol is therefore of tremendous interest, and has been the subject of a vast amount of investigation. Ergosterol, $C_{27}H_{42}O$, with three double bonds, is closely related to cholesterol, $C_{27}H_{46}O$, with one double bond. Heilbron and his associates (1) accepted the cholesterol formula proposed by Wieland (2, 3) as the nucleus of the ergosterol structure and attempted to establish the positions of the additional double bonds in ergosterol by a study of the ultra-violet absorption spectra and the products of catalytic hydrogenation of cholesterol and ergosterol and their derivatives.

A comparison of the ultra-violet absorption spectra of ergosterol and of cholesterilene led Heilbron (1) to assume that two of the three double bonds of ergosterol occupy the same positions as do the double bonds of cholesterilene. The structure of cholesterilene (4, 5) was established as:

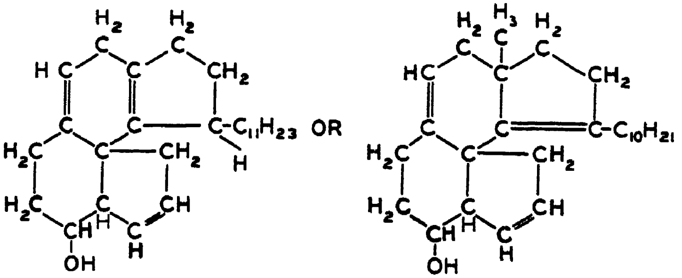


The presence in ergosterol of a double bond in the 1:2 (or 1:13) position is supported by the fact that the compound gives an immediate red color with Rosenheim's trichloracetic acid reagent (6).

In a study of the catalytic hydrogenation of ergosterol, Heilbron and Sexton (7) found a sharp break in the rate after the addition of two atoms of hydrogen in ether solution. They note that Windaus and Linsert succeeded in adding six atoms of hydrogen to ergosterol in hot glacial acetic acid containing hydrochloric acid in the presence of platinum black. Abietic acid also requires the use of hot glacial acetic acid as solvent to bring about complete saturation with hydrogen.

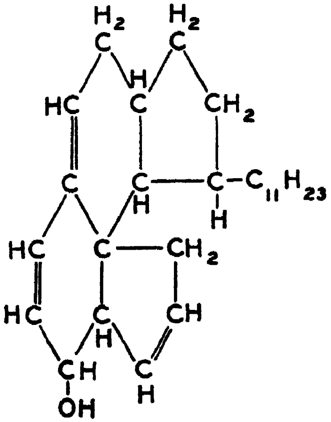
Because the resistant double bond in abietic acid joins two quaternary carbon atoms, Heilbron and Sexton suggest tentatively, by analogy, that in ergosterol the double bond which resists hydrogenation may be similarly placed. Such a position is to be found between carbon atoms 10 and 11, or, according to the work of Wieland and Vocke (3) which indicates a methyl group attached to carbon atom 11, the resistant double bond must be between carbon atoms 10 and 19.

Heilbron and his associates propose this formula for ergosterol:



Windaus (8) studied the position of the double bonds in ergosterol from the point of view of ultra-violet absorption spectra and the results of hydrogenation with sodium and ethyl alcohol. He assumed that if several unsaturated isomeric hydrocarbons or alcohols show different ultra-violet absorption spectra, the compound which absorbs most strongly in the long-wave region probably contains conjugated double bonds, and further, that the presence of conjugated double bonds is extremely probable in a compound which, in contrast with its isomer, takes up two atoms of hydrogen when treated with sodium and alcohol.

Ergosterol is transformed to dihydro-ergosterol by treating with sodium and alcohol; none of the isomers investigated was hydrogenated by this treatment. Ergosterol shows its chief absorption at 2800 Å., isoergosterol at 2470 Å., and ergosterol D at 2400 Å. Windaus therefore argues that ergosterol most probably contains a system of conjugated double bonds which, judging from the type of hydrogenated compounds formed, are in two different rings. He proposes this structure:

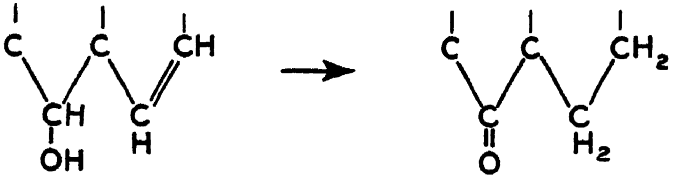


THE NATURE OF VITAMIN D

The nature of the change undergone by ergosterol when it is converted to vitamin D is not known. The opinions for which the most convincing evidence is offered are (*a*) that vitamin D is an alcohol isomeric with ergosterol and (*b*) that vitamin D is a ketone.

Windaus and his collaborators believe that vitamin D is an alcohol isomeric with ergosterol. Windaus and Linsert (9) consider four possibilities for isomerism:

1. A transfer of two hydrogen atoms from the secondary alcohol group to a double bond. A change of this sort actually takes place when cholesterol or sitosterol is heated to 220° C. with nickel.



They determined that the alcohol group of ergosterol is not decreased as a result of irradiation and concluded that this first possibility is untenable.

2. A double bond may form a ring linkage as a result of irradiation. According to the results of titration with perbenzoic acid and of catalytic hydrogenation, the number of double bonds in ergosterol does not appear to be altered by irradiation.

As isomerization possibilities there remain:

3. A rearrangement of the position of the double bonds.
4. A steric rearrangement at the secondary alcohol group, at the double bonds, or in some other place in the molecule.

Several of these changes may take place, either simultaneously or in succession, but in any case the nonprecipitability with digitonin of the compound or compounds resulting from irradiation indicates a steric change at the secondary alcohol group.

Windaus also states that since irradiation isomerizes dehydroergosterol, it seems reasonable to assume a similar change for the closely related ergosterol. Further investigations of Windaus and his co-workers have strengthened their conviction that vitamin D is an isomer of ergosterol containing three double bonds and a secondary alcohol group (10, 11).

Bills and his associates (12) are of the opinion that vitamin D is an isomer of ergosterol. They state that the earliest photochemical decomposition product of vitamin D exhibits an absorption and an extinction coefficient which apparently identify it with isoergosterol. As evidence of the importance of the alcohol group in activation, Bills notes that although ergosterol-isoergosteryl ether shows the absorption bands typical of ergosterol it can not be activated (13).

Bills and Brickwedde (14) activated cholesterol at —183° C., the temperature of liquid oxygen. Most bimolecular chemical reactions, especially oxidations, are markedly inhibited at very low temperatures, while the rates at which photomolecular changes take place decrease more slowly with decreasing temperature. The fact that activation is possible at —183° is regarded by these investigators as strong confirmation of the evidence of Windaus that activation is the result of isomerization.

On the basis of ultra-violet absorption spectra studies, Reerink and van Wijk (15, 16) also conclude that vitamin D is an isomer of ergosterol.

On the other hand, Heilbron, Morton and Sexton (1) state, "It is also unlikely, in view of the total alteration of the type of selective (ultra-violet) absorption that the reaction is due simply to stereo-chemical changes in the molecule, for cis- and trans-isomerides usually differ in extinction rather than in the general shape of the absorption curve."

These authors observed a similarity in the absorption spectra of irradiated ergosterol and of cholestenone and suggested that since cholesterol readily undergoes intra-molecular rearrangement leading to the formation of cholestenone, the conversion of ergosterol to the vitamin may follow a similar course, giving rise to ergostatrienone or a partially hydrogenated ketone derivative. These arguments are in part based on the opinion, now believed to be erroneous, that vitamin D is associated with an absorption band having a maximum at 2470 Å.

A color test for vitamin D has not yet been found. The color reactions so far proposed, however, suggest the presence in irradiated ergosterol of an aldehyde or a ketone, which, of course, is not necessarily the vitamin (17, 18).

Rosenheim and Adam (19) measured the monomolecular surface films on water of numerous sterols and sterol derivatives. The film of irradiated ergosterol strikingly resembles the films of certain ketone derivatives. The film of a very potent resin obtained by evaporating the filtrate in vacuo after precipitating unchanged ergosterol, in particular, resembles that of keto-cholesterilene. The ultra-violet absorption spectra of these two substances are very similar.

These authors suggest that irradiation may induce intra or intermolecular dehydrogenation of the CH(OH) group, the double bond of the same or of a second molecule functioning as the hydrogen acceptor. This reaction would lead to the formation of ketones with two or three double bonds, of which the former is isomeric with keto-cholesterilene and with ergosterol. They note that ketones possess the known chemical characteristics of vitamin D; as ketones they are unable to combine with digitonin, as enols they may form esters.

While the evidence presented indicates the presence of ketone derivatives among the products of irradiation, there is no evidence that these ketones constitute the biologically active portion of irradiated ergosterol.

THE RELATION OF INFRA-RED ABSORPTION TO CHEMICAL CONSTITUTION

Definite infra-red absorption bands are manifestations of definite energy relationships. Drude (20), from a conception of vibrating charged particles, worked out a mathematical relationship between given wave-lengths in the ultra-violet and corresponding wave-lengths in the infra-red spectrum. Since the frequency of electro-magnetic oscillations of infra-red radiations is slow compared with the rate of motion of electrons in atomic orbits, resonance emission and absorption can occur only for charged masses moving at a velocity much less than that of the electrons, i. e., for ions, atoms, or molecules. The infra-red absorption spectrum, therefore, is characteristic of the structural groups of the molecule.

Bjerrum (21) suggested that in addition to the frequencies due to the vibrations of charged ions and atoms, a molecule possesses a frequency due to its rotation, and that this rotational frequency accounts for the second, and apparently quite distinct, series of bands in the far infra-red spectrum.

The infra-red spectrum thus falls naturally into two regions, that in the near infra-red, the "vibration" or "rotation-vibration" spectrum, and that in the far infra-red, the "pure rotation" spectrum. Lecomte (22) states that the bands of the vibration spectrum characteristic of structure are found at wave-lengths rarely exceeding 14μ, while those of the rotation spectrum do not occur below 8 to 10μ, rarely below 14μ.

For purposes of classification, the near infra-red is usually considered as the region extending from 0.8μ, the extreme of the visible red, to 23μ in the infra-red. This region can be explored by the use of suitable prisms. The far infra-red extends from 23μ to about 300μ.

EXPERIMENTAL

The molecule of ergosterol is large and complex, but it contains only carbon, hydrogen and oxygen and these in relatively few different types of linkages. The complexity of infra-red absorption spectra increases with the number of different atoms or groupings in the absorbing molecule rather than with the general complexity or size of the molecule itself. The literature contains data giving the positions of bands identified with the alcohol and ketone groups and the relationship of the spectra of isomeric compounds. An investigation of the spectra of ergosterol and irradiated ergosterol and of isomeric and ketonic derivatives could reasonably be expected to throw some light on the nature of vitamin D.

The spectra of a number of derivatives which have been prepared for this study will be presented in a later paper. This preliminary discussion will be confined to the infra-red spectra between 0.8μ and 3μ of ergosterol and irradiated ergosterol.

SPECTROGRAPHIC EQUIPMENT

The spectrometer is a Hilger type D83, equipped with interchangeable prisms. A Hilger type F110 thermopile is used. The source is a Globar rod, of carborundum and graphite. This is heated by a current, maintained constant at 8 amperes, from a 110-volt storage battery. The housing of the rod is water-jacketed to eliminate stray heat radiations. The Globar rod spectrum is free from ultra-violet, so that activation of ergosterol by ultra-violet could not have taken place during the absorption measurements.

The galvanometer is a modified Einthoven type, built in this laboratory, after the design of Fardon (23). The silvered quartz fibre is practically free at the bottom, in contrast with the usual Einthoven design, electrical contact being made by a 1 mm. horizontal strip of the thinnest gold leaf obtainable. The most favorable fibre resistance under the conditions of the experiment was found to be about 100 ohms. With such a fibre, the instrument has a maximum sensitivity of 10^{-11} amperes per division. The field coil of the galvanometer is energized by the current from a 100-volt storage battery.

The absorption cells are brass tubes—2, 5, and 10 cm. long—with quartz windows cemented against the ground ends of the tubes. A slide in front of the entrance slit of the spectrometer holds two identical cells (one containing the solvent and the other the solution) in such a way that either one can be moved quickly and precisely into position in the path of the rays.

PROCEDURE

The entrance and the exit slits are adjusted to the same width. In this investigation a slit width of 0.02 inches was used.

The drum of the spectrometer is set at a given wave-length, and the galvanometer fiber adjusted to zero. The transmission is then read through the solution and through the solvent. The ratio of the readings is assumed to represent the fraction of the radiations transmitted by the dissolved substance in that particular wave-band. This assumes that the galvanometer readings are linear, and that there are no deviations due to outside disturbances, fluctuations of source intensity or of galvanometer field current, or variations in cells or positions of cells.

At a sensitivity of 10^{-9} divisions per ampere, readings of 30 to 50 divisions can be obtained with a reading error of about 2 per cent.

ERGOSTEROL

All of the ergosterol used was recrystallized from a mixture of alcohol and benzene (3:1) until a specific rotation of —130° to —132° was attained. Bills (24) states that "ergosterol of exceptional purity shows $[\alpha]_D^{20} = -132°$."

IRRADIATION

The purified ergosterol was dissolved in ether, 5 mg. to 1 cc, and irradiated in sealed quartz tubes at a distance of 12 cm. from a mercury arc screened by a filter which cut off wave-lengths shorter than about 2960 Å. Unchanged ergosterol was estimated by precipitation with digitonin.

POTENCY

X-ray pictures show that 0.000025 mg. a day of ergosterol irradiated with wave-lengths longer than 2960 Å. until approximately 50 per cent of the ergosterol is changed is curative for ricketic rats fed the McCollum diet. Preliminary experiments indicate that 0.00001 mg. of this irradiated ergosterol is curative, but the rats were destroyed by a fire before the test was concluded and smaller dosages have not yet been tested with a sufficiently large number of rats.

TABLE I

Proportion of Various Preparations of Ergosterol Changed by Long-Wave Irradiation as Estimated by Digitonin Precipitation

Ergosterol Preparation	Rotation $[\alpha]^{20}_{D}$	Per Cent Changed by Irradiation Using 2960 Å. Filter					
		1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.
I	129.8	....	.....	.....	....	.....	65.5
II	131.2	...	....	....	...	.	56.35
III	131.7	30.15	36.65	47.7	53.0	.	62.4
IV	131.6[1]	30.3	38 5	46.4	48.5	54.0	.
V	131.6	34.0	41.2	42.4	46.0	48.5	
VI	131.6	29.5	38.0	45.1	48.0	53.0	
VII	131.6	27.1	34.2	42.3	49.0	52.3	.
VIII	131.6[1]	26 2	37.0	44.0	47.0	53.0	. .

[1] Preparations IV and VIII represent the same sample of purified ergosterol.

SOLVENTS

The selection of a suitable solvent for the solution to be examined in the infra-red region proved difficult. Ether, alcohols, benzene, hexane, and cyclohexane do not decompose ergosterol, but they all absorb strongly at critical points or they become opaque below 3μ. A solvent which does not contain the C-H bond is required. Both carbon disulphide and carbon tetrachloride were used.

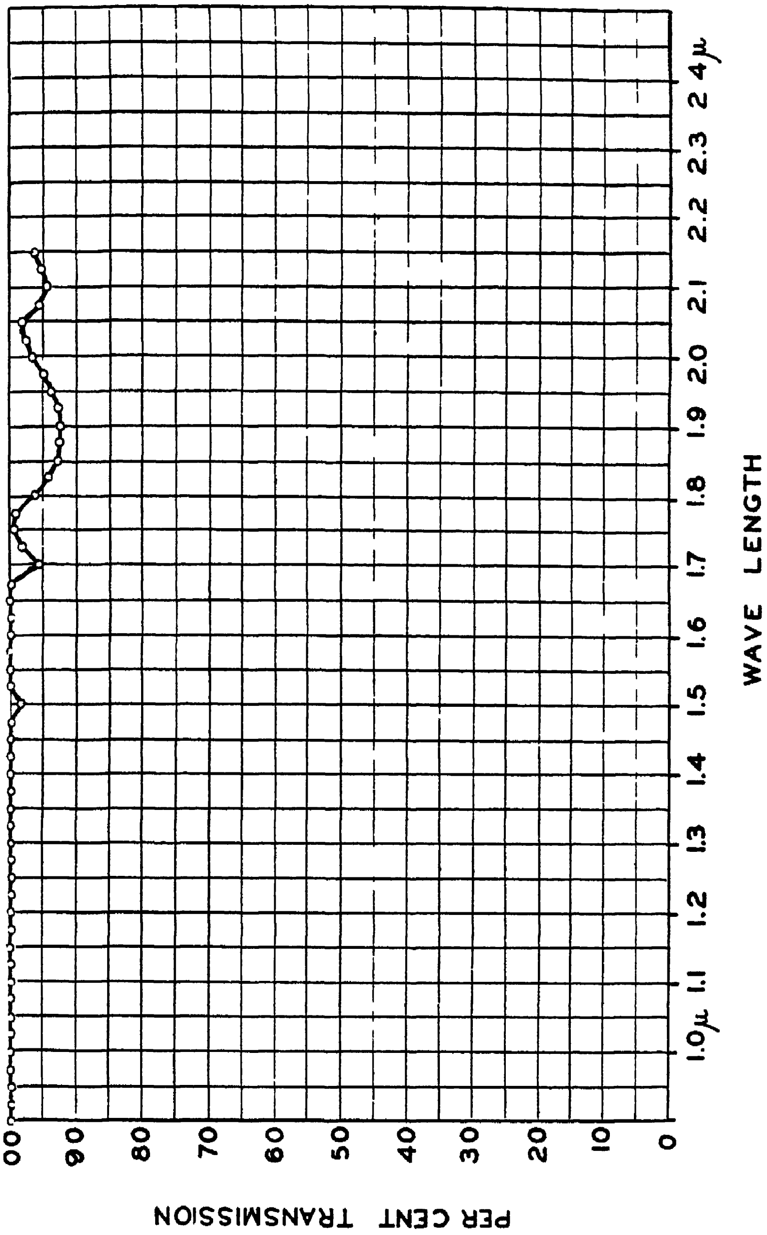


Fig. 1—Infra-Red Absorption of Carbon Tetrachloride

Carbon tetrachloride is excellent from the standpoint of transmission (Figure 1), but solutions of ergosterol in this solvent become yellow within sixteen to twenty hours. Solvents are still being investigated, but with proper precautions carbon tetrachloride is satisfactory, and was used for all of the curves presented in this paper.

Most of the carbon tetrachloride used was purified and redistilled. After absorption curves of purified and C.P. carbon tetrachloride had been found to check, and no difference in the behavior of solutions of ergosterol in either one could be detected, C.P. solvent was used. It was tested for free chlorine and for chloroform before each solution was made, and fresh solutions were made for each curve. During the first year of the investigation, the solutions were again tested after the curve was made; later, only occasional samples were tested.

The fact that the different curves for ergosterol and for ergosterol irradiated with wave-lengths longer than 2960 Å. do not vary greatly may be taken as evidence that appreciable decomposition does not occur during the five or six hours required for the readings. The curves of ergosterol exposed to the unfiltered radiations of a mercury arc lamp can not be duplicated, probably because of the presence of unstable products of irradiation.

SPECTROGRAPHIC RESULTS

From a large number of curves of the various materials investigated, a few typical ones have been chosen for discussion here.

Average curves showing the infra-red absorption of pure ergosterol and of ergosterol irradiated with wave-lengths longer than 2960 Å. for different periods of time are shown in Figure 2.

For comparison, the spectra of ergosterol irradiated with unfiltered ultra-violet light (Figure 3) and of *a*-isoergosterol (Figure 4) and ergostadienone (Figure 5) are included. The infra-red absorption bands of ergosterol irradiated with unfiltered ultra-violet light vary so greatly that fairly typical individual curves are shown in Figure 3, rather than average curves.

The isoergosterol was prepared by treating an alcohol solution of ergosterol with sulphuric acid according to the method of Heilbron (25). The ergostadienone was made by heating ergosterol with nickel, as described by Windaus and Auhagen (26).

THE CORRELATION OF ABSORPTION BANDS AND LINKAGES

The recurrence of certain features among the infra-red spectra of chemically related compounds was noted by Abney and Festing (27) in 1881, but the work of correlating bands and linkages is still far from complete. A single band is rarely significant; series of bands are definitely identified with chemical linkages. The bands below 3μ are in most cases overtones of fundamental frequencies which occur at longer wave-lengths. For this reason, although the bands are crowded and of low intensity, this region is significant because a number of bands of a given series are present.

THE C-H LINKAGE

Ellis (28, 29, 30) ascribes a series of bands of decreasing intensities occurring near 2.3μ, 1.7μ, 1.38μ, 1.17μ, 1.03μ and 0.9μ to the characteristic oscillation of the C-H linkage. He believes that these bands represent the third to the seventh harmonics of a fundamental frequency occurring near 6.9μ. The C-H linkage has been investigated by many workers, but the positions of most of the absorption bands found agree fairly closely with those in the series of Ellis.

THE ALCOHOL GROUPS

The alcohol groups have been investigated by Weniger, Puccianti and Coblentz, and more recently by Lecomte and Sappenfield. These investigators do not always agree as to the positions of bands assigned to the OH linkage.

Lecomte (31, 32) identifies two strong bands, definitely separated, at 3μ and 3.4—3.5μ. Bands which are probably due to the absorption of the OH linkage occur at 4.3—4.6μ, 4.8—4.9μ, 5.2—5.3μ, 5.98—6.12μ and 6.98—7.2μ. Characteristic differences in the spectra of primary, secondary, and tertiary alcohols occur beyond 6.9μ.

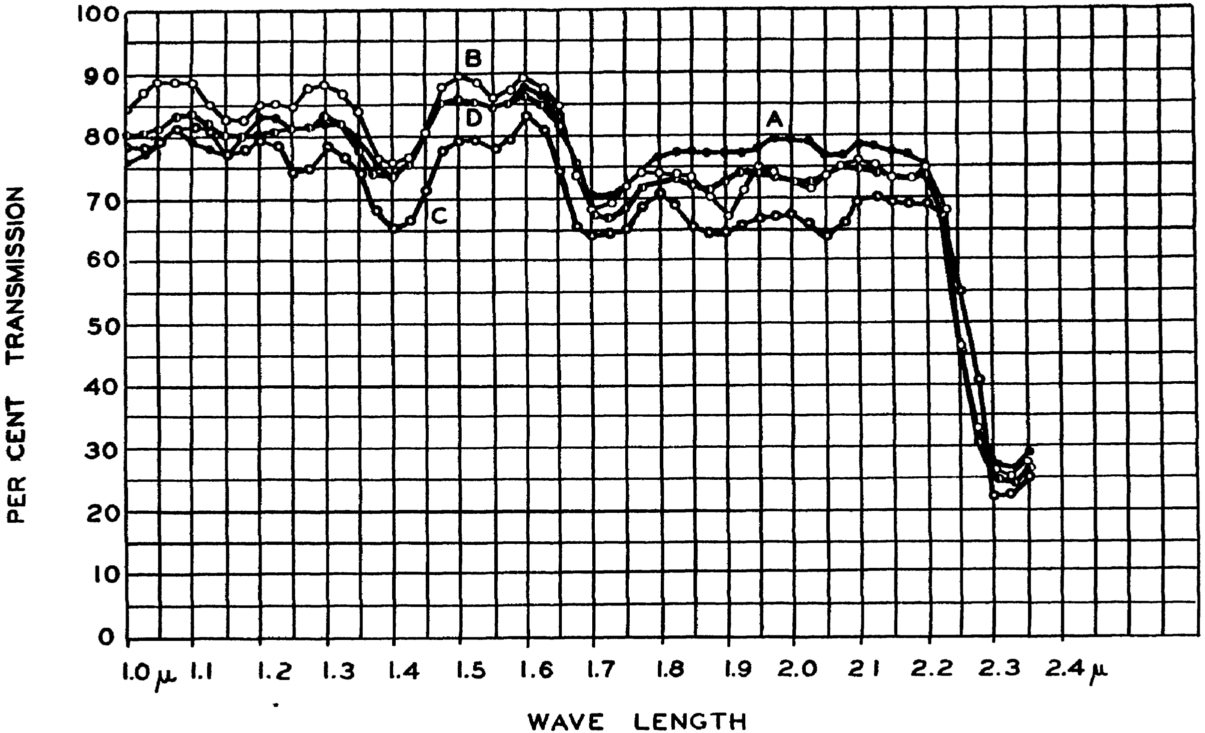


Fig. 2—Infra-Red Absorption of Ergosterol Irradiated through 2960 Å. Filter
Curve D, unirradiated; curve C, irradiated 1 hr.; curve B, irradiated 3 hrs.; curve A, irradiated 5 hrs.

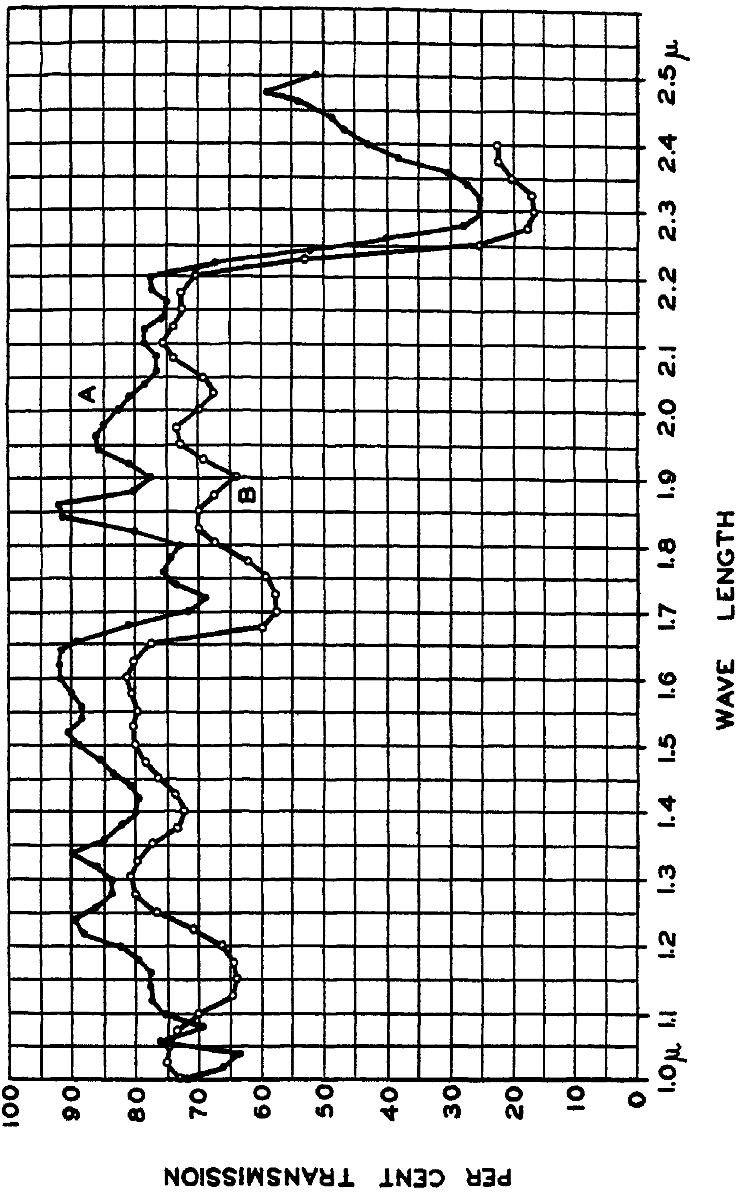


Fig. 3—Infra-Red Absorption of Ergosterol Irradiated with Full Ultra-Violet
Curve B, irradiated 15 min; curve A, irradiated 30 min.

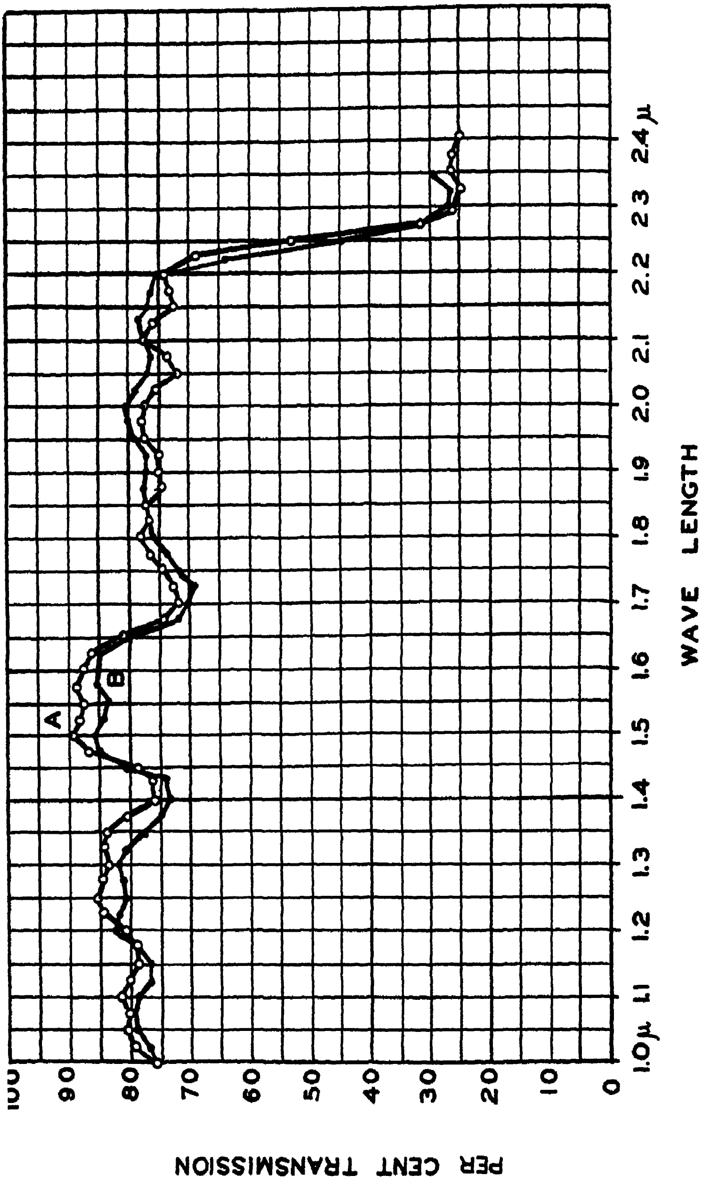


Fig. 4—Infra-Red Absorption of Ergosterol (curve B), and *α*-Isoergosterol (curve A)

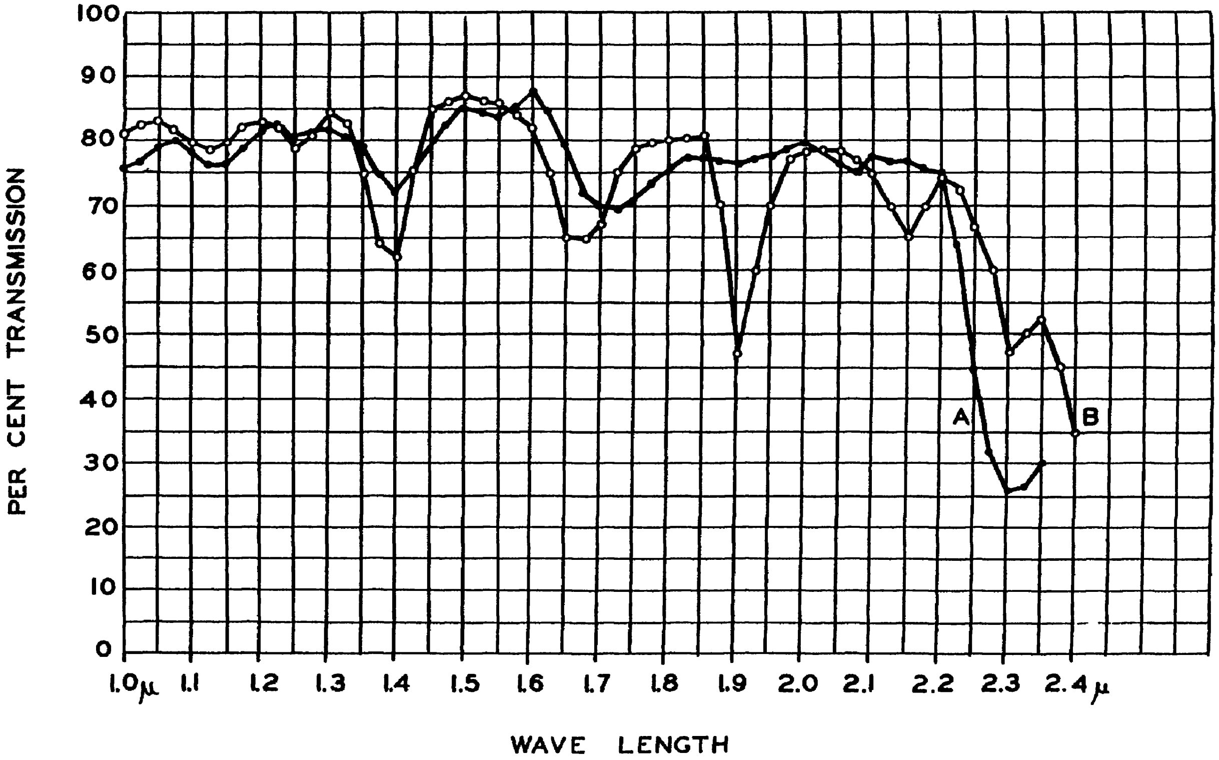


Fig. 5—Infra-Red Absorption of Ergosterol (curve B), and of Ergostadienone (curve A)

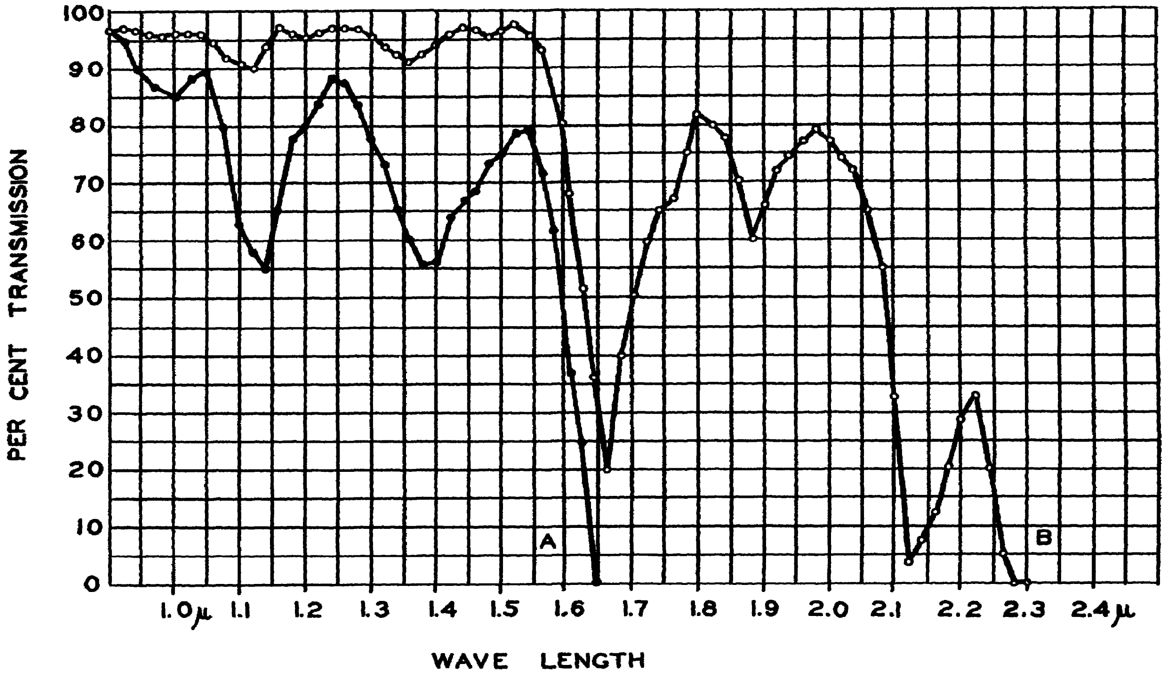


Fig. 6—Infra-Red Absorption of Acetophenone
Curve A, 20 mm. cell; curve B, 2 mm. cell

Coblentz (33) found a shallow band at 2.85μ which is characteristic of alcohols; he also identifies with the OH group the shallow band at 2.08μ to 2.14μ in Puccianti's curves.

According to Sappenfield (34), the absorption bands due to the OH group occur near 6μ, 3μ, 1.55μ and 1.025μ. Since bands at 6μ and 3μ are found in the spectrum of water, they may be assigned to the OH group with some assurance. The strong band near 1.55μ in the spectra of primary, secondary, and tertiary alcohols is undoubtedly due to the OH group. Sappenfield also relates weak bands near 1.02μ and 1.69μ to this group, although these bands do not appear in the spectrum of every alcohol studied.

THE CARBONYL GROUP

From the results of a study of twenty-five aldehydes and ketones, Lecomte (35) concludes that a band between 5.9μ and 6.03μ characterizes the carbonyl group. The first overtones of this band occur near 2.9μ and 3.5μ. Ellis (36) believes that Lecomte's values are consistently too high and that the fundamental band appears at 5.8μ.

A summary (36) of the results of Ellis, Lecomte, Henri, Coblentz, Smith and Boord, and Bennet and Daniels shows that a band near 1.9—1.97μ is present in the spectrum of every carbonyl compound examined; practically every one shows bands near 5.8—6.0μ and 2.12—2.62μ; and many near 2.9μ and 1.25μ. Ellis believes that these investigations as well as his own results, establish at 5.8μ the fundamental band due to the absorption of the $=C=O$ linkage, with overtones near 2.9μ, 1.9μ, 1.45μ, 1.16μ and 0.97μ.

Acetophenone (Figure 6) shows the intense absorption near 1.9μ and 1.4μ which is characteristic of ketones.

THE STEROL NUCLEUS

Conjugated ring compounds have not been extensively investigated and no bands have as yet been definitely associated with the sterol nucleus itself.

TABULAR SUMMARY

The principal bands associated by different workers with the C-H, alcohol, and ketone linkages are summarized in Table II.

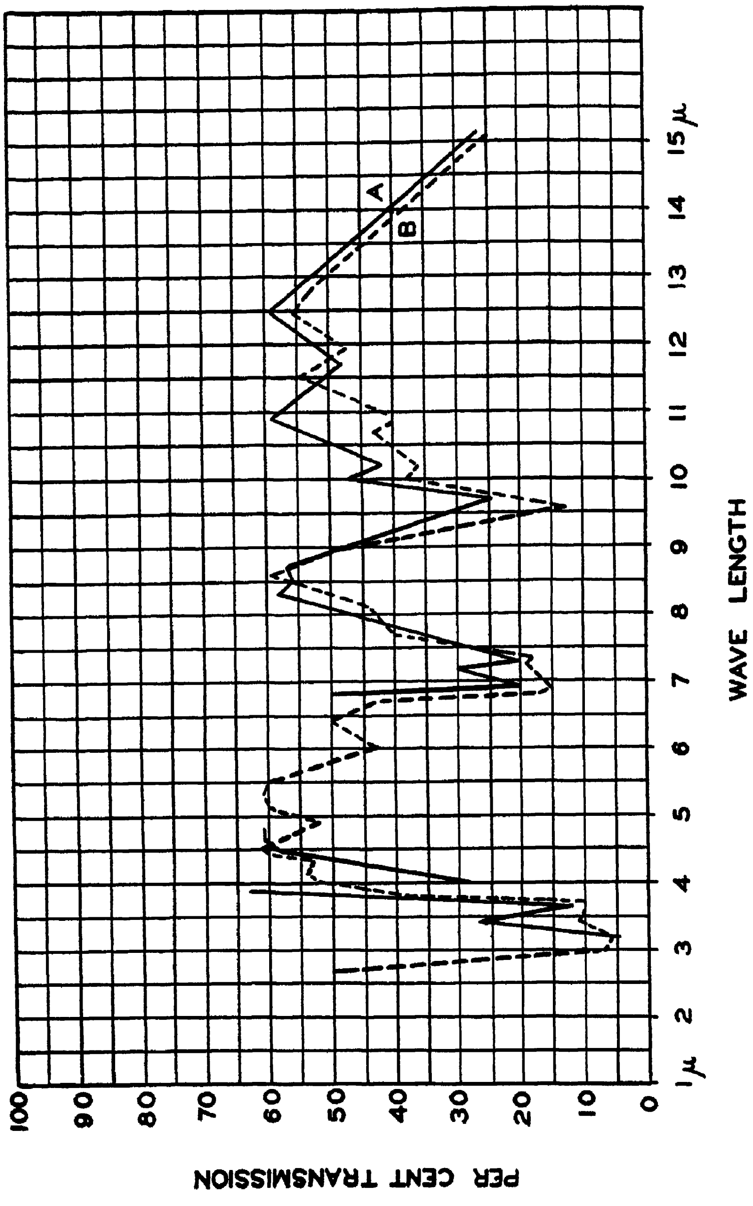


Fig. 7—Infra-Red Absorption of Butyl Alcohol (curve A), and Isobutyl Alcohol (curve B), after Lecomte (32)

ISOMERS

A study of isomeric compounds shows the influence of the arrangement of atoms in the molecule. Isomers containing different groups, for instance an ether isomeric with an alcohol, have distinct spectra, while stereoisomers such as *d*- and *l*-pinene have identical spectra. Coblentz (37) states that the spacial arrangement of atoms in the molecule has no effect upon the infra-red spectrum.

TABLE II

The Principal Absorption Bands Associated with the CH, OH, and =C=O Linkages.

LINKAGE	WAVE-LENGTHS OF PRINCIPAL BANDS (REFERENCES IN PARENTHESES)						
C-H	0.9	1.02	1.17	1.4	1.7	**2.3**	(E1)
OH		1.025 (S)		1.55 (S)	2.08-2.14 (P)(C)		2.95 (C)
					1.69	1.94 (S)	
=C=O	*0.97*		1.16	**1.45**	1.9	2.12-2.62 (E2)	
							2.9(L)

Bold Face indicates intense band.
Italics indicate faint band, not present in all compounds examined.
E1—Ellis (28, 29, 30).
E2—Ellis (36).
C—Coblentz (33).
L—Lecomte (35).
P—Puccianti (33).
S—Sappenfield (34).

Lecomte (38, 39) reviews studies of isomers, particularly of esters isomeric with organic acids. Such isomers show different absorption curves, while butyl and isobutyl alcohols have very similar curves (Figure 7). Lecomte finds that the chief absorption bands of isomers occur at identical positions while less intense bands characteristic of the individual compound occur in different positions.

THE ABSORPTION SPECTRA OF ERGOSTEROL AND IRRADIATED ERGOSTEROL

The infra-red absorption bands of ergosterol near 1.0μ, 1.15μ, 1.4μ, 1.7μ and 2.3μ are quite evidently members of the series of bands associated with the C-H linkage.

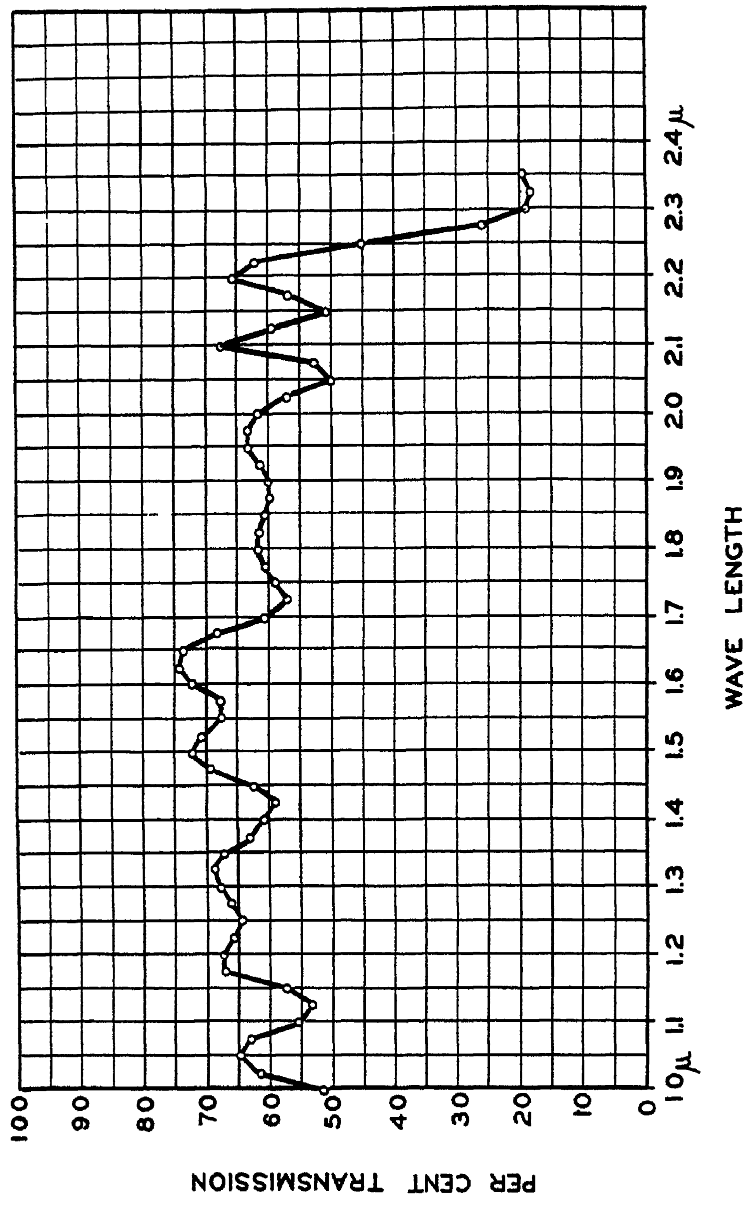


Fig. 8—Infra-Red Absorption of Ergosterol Irradiated for 6 Hours through 2960 Å. Filter

Those at 1.55μ and 2.05μ are identified with the alcohol group. The band near 1.99μ and the faint band near 1.25μ, which appear both in the spectra of ergosterol and irradiated ergosterol, are probably associated with the alcohol group since bands in these positions are found in the spectrum of almost every alcohol studied by Sappenfield. It must be noted that these bands have also been assigned to the carbonyl group by Ellis.

The band near 2.15μ is possibly identified with the sterol nucleus. This band appears in the spectra of all of the ergosterol and cholesterol derivatives thus far examined. Benzene compounds absorb near 2.18μ.

The infra-red absorption spectra of series of samples irradiated through the 2960 Å. filter for periods ranging from one to six hours, with 26 to 65 per cent of the ergosterol changed, gave no evidence of decrease in the absorption bands attributed to the alcohol group nor of the development of bands associated with the ketone group.

Sample I, irradiated for six hours, with about 65 per cent of the ergosterol changed, shows bands at 1.55μ and 2.05μ in undiminished intensity, indicating the persistence of the alcohol group (Figure 8). The fundamental ketone band occurs at 5.8μ with overtones at 1.9μ and 1.4μ in the region investigated. Unfortunately these overlap bands assigned to the C-H linkage, and definite conclusions must await an investigation of spectra beyond 5.8μ. However, the absorption of long-wave irradiated ergosterol is not increased at 1.9μ and 1.4μ as would be expected if a ketone group had been found as a result of irradiation.

In almost every case, curves of ergosterol irradiated with unfiltered ultra-violet light show a fairly intense peak at 1.9μ. Chemical and biological investigations and studies of the ultra-violet spectra have shown that the products of irradiation with long ultra-violet wave-lengths are different from the products of short-wave irradiation. The differences in the infra-red spectra suggest that a ketone derivative may be the result of irradiation with ultra-violet light of short wave-length. A large part of the evidence for the presence of a ketone in irradiated ergosterol is based upon studies of short-wave irradiated preparations.

Ergostadienone, a ketone derivative of ergosterol, shows intense peaks at 1.9μ and 1.4μ and a shift of the 1.7μ band (Figure 5). The general form of the curve of ergosterol irradiated with ultra-violet light of wave-lengths longer than 2960 Å. closely resembles that of pure ergosterol and isoergosterol; it does not resemble the curve of ergostadienone.

A summary of the principal bands found in ergosterol and ergosterol derivatives is given in Table III.

TABLE III

Infra-red Absorption Bands of Ergosterol and Ergosterol Derivatives

Unirradiated Ergosterol	Ergosterol Irradiated Through 2960 Å. Filter —— 50% Changed	Ergosterol Irradiated With Unfiltered Ultra-Violet —— 47% Changed	Isoergosterol	Ergostadienone
1.0μ	1.0μ	1.0μ	1.0μ	1.0μ
				1.15
1.25	1.25	**1.25**	*1.25*	**1.25**
1.4	1.4	**1.4**	1.4	**1.4**
1 5	1.5	*1.5*	1.5	....
1.7	1.7	1.7	1.7	**1.65**
1.9	*1.9*	**1.9**	1.9	**1.9**
2.0	2.05	.... .	2.05	....
2.1	2.15	2.1	2.15	2.15
2.3	2.3	2.3	2.3	2.3

Bold Face indicates intense band.
Italics indicate feeble band.

CONCLUSIONS

This preliminary investigation indicates that the alcohol group persists in ergosterol irradiated with wave-lengths longer than 2960 Å., while bands usually associated with the ketone group do not appear. The resemblance of the curve of long-wave irradiated ergosterol to that of pure ergosterol and of isoergosterol suggests that the product of irradiation is an alcohol isomeric with ergosterol.

It is planned to extend this investigation farther into the infra-red region in order to study the more intense and widely separated fundamental bands of a number of ergosterol and cholesterol derivatives.

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VITAMIN D—A REVIEW

PART I—ULTRA-VIOLET ABSORPTION SPECTRA IN RELATION TO VITAMIN D.

SECTION C

BY JOHN R. LOOFBOUROW

PREVIOUS SECTIONS

Part I, Sections A and B, appeared in *The Bulletin of Basic Science Research*, 3 (1931), page 101. These sections dealt with "Early Attempts to Correlate Absorption Spectra and Vitamin D Activation," and "Ergosterol as the True Precursor of Vitamin D."

SUMMARY OF PART I, SECTION C

C. The Ultra-Violet Absorption Spectra of Oils

Lewkowitsch (45) investigated the ultra-violet absorption spectra of a number of oils, mainly with the purpose of differentiating between similar oils. Heilbron, Kamm, and Morton (46) studied the absorption spectra of various oils from the standpoint of their ergosterol and vitamin D content. Woodrow (48) detected the three ergosterol bands in cod-liver oil. Takahashi, Nakamiya, Kawakami, and Kitasato (49) associated absorption at 3200 Å. with vitamin A. Peacock (50) found a decreased absorption in cod-liver oil so irradiated as to lose its vitamin A potency. Morton and Heilbron (51), from an examination of many oils, came to the conclusion that vitamin A is characterized by a band in the 3200-3280 Å. region. They found a correlation between the biological, colorimetric, and spectrographic tests. Rosenheim and Webster (53) criticized the conclusions of Morton and Heilbron (51) because dehydroergosterol has a strong absorption band in the 3200-3280 Å. region. Drummond and Morton (55) confirmed the findings of Morton and Heilbron (51) regarding correlation between absorption at 3280 Å. and the colorimetric and biological tests for vitamin A. The criticism of Rosenheim and Webster (53) was answered by Morton, Heilbron, and Spring (56), who state that the dehydroergosterol absorption spectrum exhibits a fine structure not present in the spectrum of vitamin A. Definite evidence of the growth-promoting properties of carotene was advanced by Euler, Euler, and Helström (57). Using more highly purified carotene, Duliere, Morton, and Drummond (58, 59) failed to obtain growth-promotion, nor could they find an ultra-violet band in the 3200-3300 Å. region for carotene. They attributed the positive results of Euler, Euler, and Helström (57) to contamination of carotene with vitamin A. On the other hand, Moore (65, 66), Collison, Hume, Smedley-MacLean, and Smith (67), Kawakami and Kimm (68), and Euler, Euler, and Karrar (69) found carotene to promote growth in small doses. Hume and Smedley-MacLean (70) traced the negative results of Duliere, Morton, and Drummond (58, 59) to the use of ethyl oleate as a solvent for the carotene, this solvent resulting in rapid oxidation of the carotene. Capper

(71) compared the ultra-violet absorption and biological activity of carotene and a vitamin A concentrate, and concluded that the biological activity of carotene could not be accounted for by contamination with a vitamin A having 3280 Å. absorption. Moore (72, 73, 74) cited evidence to the effect that carotene is changed to vitamin A in the livers of animals. The ultra-violet spectra of the liver oils from Moore's rats are discussed by Capper (75), who found a 3250 Å. band in the oils from rats fed carotene but not in those from rats on an A-free and carotene-free diet. Drummond, Ahmad, and Morton (76) also came to the conclusion that the negative results of Duliere, Morton, and Drummond (58, 59) were due to the use of ethyl oleate as a solvent for carotene. They confirmed the results of Capper (75) regarding the spectra of liver oils from rats fed carotene. Morton, Heilbron, and Thompson (77) discuss spectrographic and color tests for vitamin A in various materials. Morton and Heilbron (78, 79) describe the ultra-violet absorption of butter from the standpoint of its carotene and vitamin A content. Morton, Heilbron, and Thompson (80) published data regarding the spectra of liver oils and concentrates. They and Gillam, Heilbron, Hilditch, and Morton (81) investigated the banded spectra of acids from vitamin A-containing materials.

C. THE ULTRA-VIOLET ABSORPTION SPECTRA OF OILS

The identification of ergosterol by numerous workers as the true precursor of vitamin D, and the association of certain types of absorption with the formation of vitamin D from ergosterol, led numerous investigators to study the ultra-violet absorption spectra of oils with a view to determining their ergosterol or vitamin D content.

DETERMINATION OF ADULTERANTS BY ABSORPTION SPECTRA

Lewkowitsch (45) published a paper in 1927 in which he approached the subject from another viewpoint, that of the differentiation between similar oils, as in the determination of the adulteration of olive oil by tea seed oil, and the mixture of whale and herring oils.

The spectra of the oils were photographed (Ac4)[1] through films 0.01 inch thick between quartz plates, a few being also examined in solution in hexane in such a concentration and thickness as to correspond to 0.01 inches of undiluted oil. "As experiment showed that the absorption of the oil varied after prolonged exposure to ultra-violet light, the oil sample was renewed after two to three hours' exposure."

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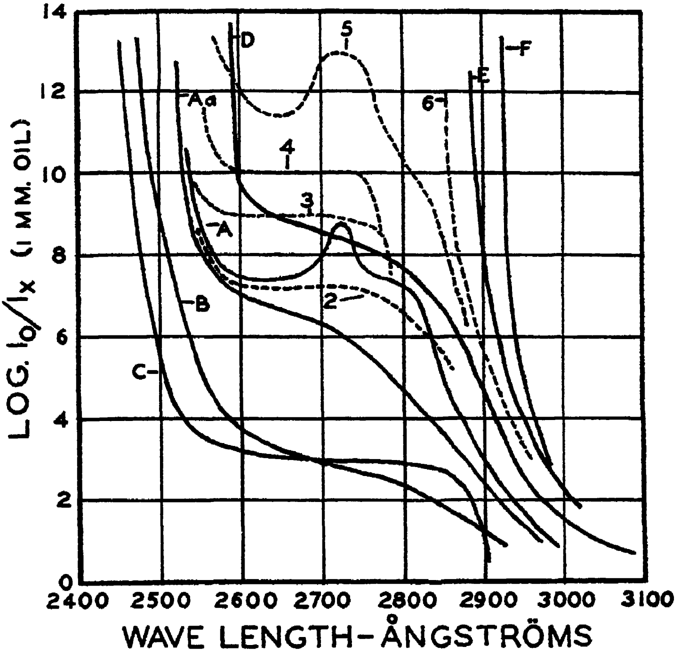
[1] These key letters refer to the spectrographic methods used by the authors of papers. An explanation will be found in the first section of this review [*Bull. Bas. Sci. Res.* 3 (1931): 104].



0.01 inch thicknesses of oil; a few samples in hexane solutions (GG III).

Fig. 21—Absorption of Olive and Tea-seed Oils [Lewkowitsch (45; 1927)]. See footnote (¹) below.

Curve *A*, greenish-yellow olive oil; curve *Aa*, same after six months' exposure to daylight in white bottle; curve *B*, golden-yellow olive oil; curve *C*, golden-yellow olive oil; curve *D*, colorless olive oil; curve *E*, oil marketed as olive oil but of doubtful origin; curve *F*, tea-seed (Susanqua) oil; curve 2, olive oil *A* containing 9.1 per cent tea-seed oil; curve 3, same but containing 16.7 per cent; curve 4, same but containing 25.0 per cent; curve 5, same but containing 33.0 per cent; curve 6, same but containing 50.0 per cent.

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[1] In order to facilitate comparison, the absorption curves of the oils shown herein have been re-plotted wherever possible in terms of Log_{10} (I_0/I_x) for 1 mm. layers of the oils. This has been done by assuming that Beer's law holds, and (in the case of oils measured in solution) that the solvent does not alter the character of the absorption. Since these assumptions may not be entirely justified, even though they make comparison more convenient, the actual conditions under which the absorption curves were obtained by the

The oils all showed general absorption, increasing rapidly toward the shorter ultra-violet. In whale oil, an absorption band at 3350 Å was found. Olive oil had a band at 2750 Å. One might identify this with one of the ergosterol absorption bands, although the author makes no such suggestion, and the results of Heilbron, Kamm, and Morton (46; 1927) as we shall see, disagree with such a viewpoint.

TABLE III

Ultra-Violet Absorption of Oils as Determined by Lewkowitsch (45; 1927).

OIL	COLOR	ABSORPTION
Olive	Greenish Yellow	Distinct band at 2750 Å.
Olive A.	After 6 mo. exposure to daylight in white glass bottle—slightly bleached by this treatment.	Band smoothed out to an an inflexion.
Olive	Golden Yellow.	Inflexion present.
Olive	Golden Yellow.	Inflexion present.
Olive	Water White.	Inflexion present.
Oil marketed as olive but of doubtful origin.	Pale Yellow; taste, flat; and not distinctively of olive oil.	No trace of inflexion in range examined.
Tea-seed Oil. (Susanqua oil.)	Golden Yellow.	Curve nearly vertical.

Note: Acid values, saponification values, and iodine values are also given in the original paper.

He examined alcoholic solutions of chlorophyl to determine whether or not traces of it might explain the presence of the 2750 Å band in olive oil. The chlorophyl solution gave a well-marked band at 4210 Å. and another at 3300 Å., but none at 2750 Å. It could not account, therefore, for the olive oil peak.

Lewkowitsch's results are reproduced in Figures 21 and 22 and Table III.

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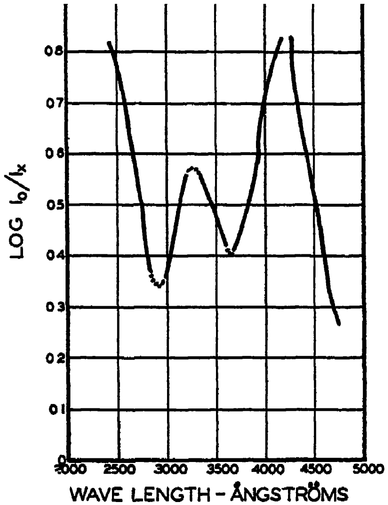
various authors, where they are stated, are recorded in small type just below the cuts. The key letters in parentheses at the ends of these cut lines are a code designating the methods of plotting curves used by the authors. A table of these key letters will be found in the first section of this review [*Bull. Bas. Sci. Res.* 3 (1931): 105].

SPECTROGRAPHIC DETECTION OF ERGOSTEROL IN OILS

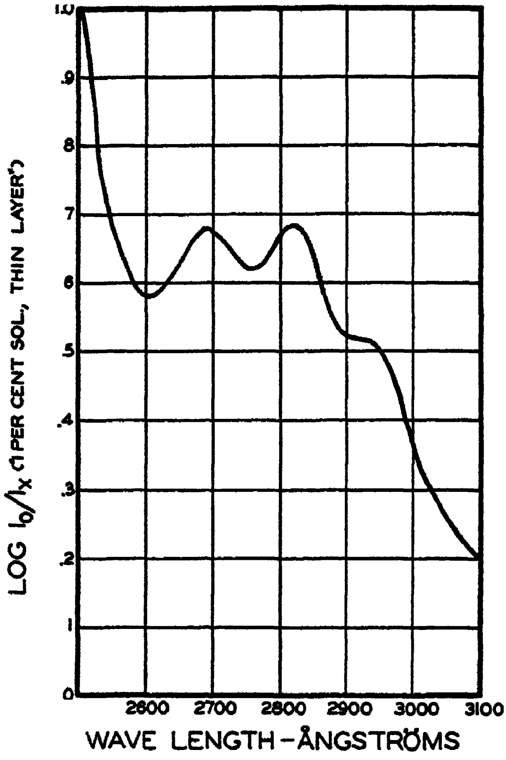
Heilbron, Kamm, and Morton (46; 1927) made a study of the absorption spectra of various oils from the standpoint of their provitamin D content. Believing that it had been sufficiently established by themselves, Pohl, Windaus and Hess, and Rosenheim and Webster that ergosterol is the only precursor of vitamin D, and knowing that various researchers had shown that vegetable oils acquire antirachitic potency upon irradiation with ultra-violet light, they thought that it would be worth while to investigate the ultra-violet absorption of these vegetable oils with a view to determining whether or not the typical bands of ergosterol at "2935, 2815, and 2700 Å." could be detected in them.



Alcohol solution, concentration and cell thickness not stated (GG). See footnote page 203.

Fig. 22—Absorption of Chlorophyl [Lewkowitsch (45; 1927)]

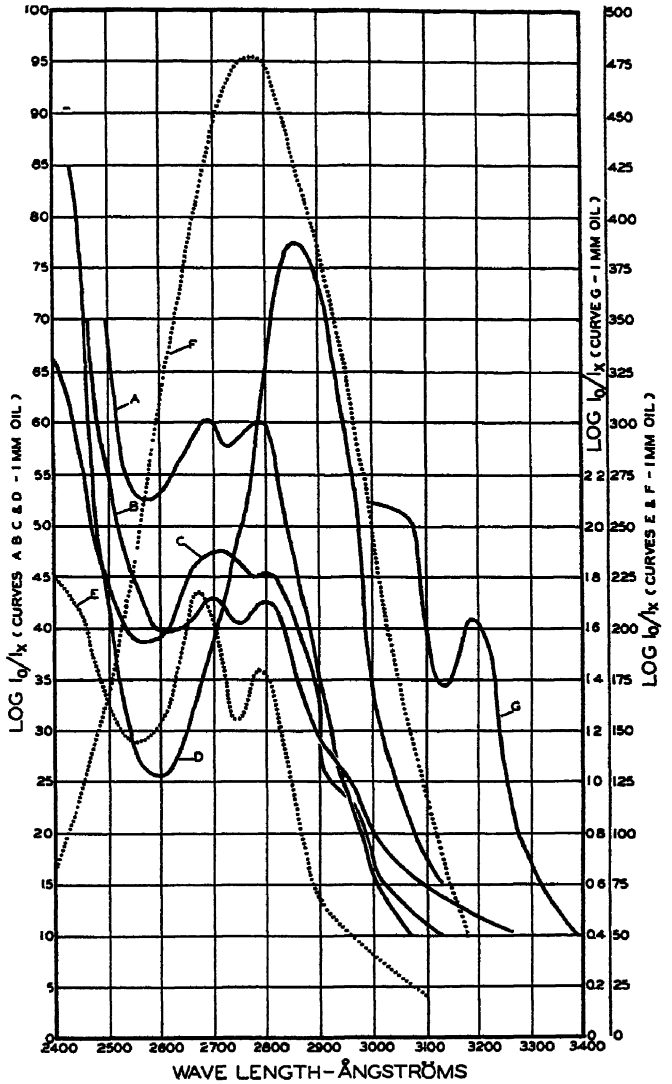
They first considered the possibility that other substances in the oils might mask the presence of the ergosterol. They reasoned that "aliphatic esters rarely if ever exhibit marked selective absorption," and since "there is no evidence that glycerides of fatty acids behave differently from esters," and in view of the fact that they themselves had shown pure cholesterol, and sitosterol "which is the chief constituent of the unsaponifiable matter of vegetable oils" to have no selective absorption, that any masking of ergosterol absorption bands in oils could be due only to the possible occurrence of unusual substances in particular oils, or to the failure of feeble amounts of ergosterol "to show themselves in the presence of the general absorption of the other constituents already mentioned." The possible effect of the particular method of extraction is also mentioned.



1.0 per cent solution, "thin layer" (GGco).
See footnote page 203.

Fig. 23—Ergosterol Dissolved in Kapok-seed Oil [Heilbron, Kamm, and Morton (46; 1927)].

They tested the spectrographic effect of the addition of small amounts of ergosterol to transparent oils, such as kapok-seed oil and cottonseed oil. A film about 0.1 mm. thick of kapok-seed



A. B. C. 1 per cent in alcohol, 2 mm. cell; D, 0.5 per cent in alcohol, 4 mm. cell; E, 5 per cent in alcohol, 1 mm. cell; F, 0.01 per cent in alcohol, 4 cm. cell; G. 0 5 mm. oil (GGccI). See footnote page 203.

Fig. 24—Absorption of Oils [Heilbron, Kamm, and Morton (46; 1927)].

Curve *A*, edible cottonseed oil; curve *B*, refined linseed oil; curve *C*, edible maize oil; curve *D*, sesamé oil; curve *E*, ground nut (Arachis) oil; curve *F*, shea butter; curve *G*, ground nut (Arachis) oil.

oil was found to be "transparent" down to 2400 Å. Addition of 0.2 per cent of ergosterol gave three absorption bands "detectable with certainty" (see Figure 23). Lesser amounts of ergosterol gave "positive results," which, however, were not always consistent. With clear samples of cotton seed oil they could detect percentages as low as 0.01 to 0.02. Thus, they concluded that the sensitivity of the method would "depend primarily upon the transparency of the oil under investigation. . . . In what follows a 'negative' test does not, therefore. *exclude* concentrations of ergosterol less than 0.2 per cent."

SPECTROGRAPHIC DETECTION OF VITAMIN D IN OILS

They were also interested in determining by spectroscopic means the presence of vitamin D in the various oils investigated, their previous work (to be discussed later) having indicated that the irradiation of ergosterol by ultra-violet light, during which vitamin D is formed, is accompanied by the appearance of a new absorption band at 2470 Å., identified with vitamin D (this point of view has since been abandoned, as we shall see). "Unfortunately very considerable concentration of the pure vitamin (of the order of 0.01 g. per 100 cc. of alcohol) is necessary to secure the detection of this absorption band. In view of the fact that such a concentration is equivalent to some thousands of doses of cod-liver oil (in respect of vitamin D) it is not surprising that the test will not serve as a criterion of the absence or presence of the antirachitic vitamin. As further indications of the limits of the spectroscopic method, it may be pointed out that cod-liver oil, the source of our original 'active' cholesterol, does not exhibit provitamin bands, although it does, of course, contain the precursor substance. A pronounced inflection, only, is shown in the same region as the ergosterol bands. It will thus be seen that the method only enables us to discriminate between oils containing relatively little ergosterol and those containing quite reasonable amounts." [1]

RESULTS OBTAINED BY HEILBRON, KAMM, AND MORTON

We have reproduced their results in Table IV and the curves of absorption which they found in Figures 23 and 24.

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[1] Woodrow, as we shall see, obtained definite ergosterol bands in cod-liver oil.

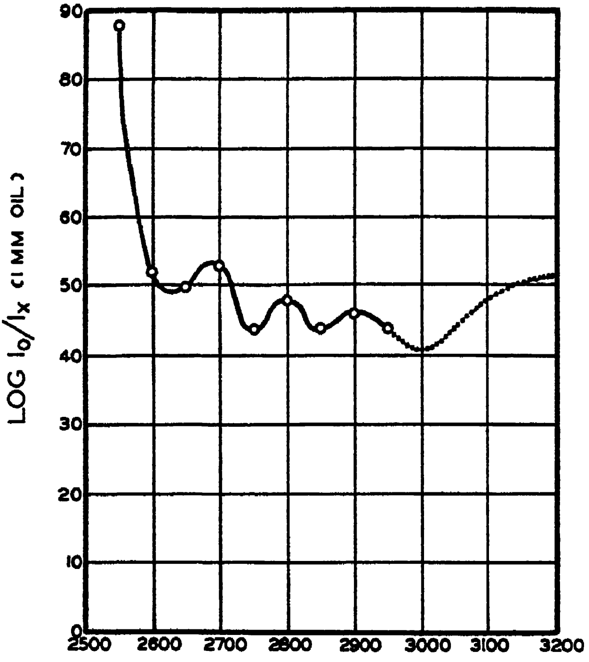
TABLE IV

Ultra-Violet Absorption of Oils as Found by Heilbron, Kamm, and Morton (46; 1927)

Oil	Ergosterol Bands	Band at or Near 3200 Å.	Remarks
Olive	—	—	Band at λmax. = 4070 Å.
Nux vomica	—	—	
Tiger nut	—	—	
Alfalfa seed	Very feeble	—	
Coconut	—	—	
Babassu	—	—	
Yeast Fat	+++++	—	Well-defined bd. λ max. = 2870 Å.
Sesame	?	—	
Kapokseed	—	—	
Rape	—	—	
Soya Bean	—	—	
Mowraseed	Inflection 2650-2800 Å.	—	
Illipi Nut	?	—	Sel. abs. λmax. = 2700 Å.
Shea Nut	?	—	Sel. abs. λmax. = 2780 Å.
Cottonseed	++	—	
Linseed	++	—	
Maize	++	—	
Ground Nut, Sample A	Inflection.	—	
" " " B	—	—	
" " " C	—	—	
" " " D	+	—	
" " " E	++	+	
" " " F	+	—	
" " " G	++	++	
" " " H	++	++	
Brazil Nut	—	—	
Palm Oil	—	—	
Palm Kernel	—	—	
Rice Meal	—	—	Well defined sel. abs. λ max. = 2765Å. No fine structure.
Pumpkin Seed	?	—	
Sunflower	—	—	
Whale	—	?	
Wool-fat	Inflection	++	
Cod-liver	?	+	
Ditto unsap.	?	+	
Extracted c. l. o.	?	++	
"Ostelin"	?	+	

DISCUSSION OF INDIVIDUAL OILS

A number of the oils investigated are discussed in detail. In addition to studying the absorption spectrum of the oil itself, they investigated the unsaponifiable fraction of cottonseed oil. The saponification yielded considerable crystalline sterol, "which was fractionally crystallized from ethyl acetate, the various fractions being examined spectroscopically." They found that the least soluble fraction contained 2.5 per cent ergosterol and the most soluble fraction 0.75 per cent. These results indicated that cottonseed oil is rich in ergosterol.



0.04 mm oil (GGI). See footnote page 203

Fig. 25—Absorption of Cod-Liver Oil [Woodrow (48; 1928)].

While sesame, illipi, shea-nut, and pumpkin-seed oil all showed "well-defined selective absorption in the middle ultraviolet," they did not exhibit the characteristic ergosterol peaks, and they felt therefore that there was no good reason for associating this absorption with the presence of ergosterol. A preliminary examination of the unsaponifiable fraction of shea nut oil failed to give an indication of the presence of ergosterol.

Of yeast fat, they say, "As would be expected from the work of Smedley-MacLean, and Thomas (47; 1920), who found it to contain as much as 40 per cent ergosterol, this fat gave by far the most pronounced absorption of all the oils examined."

Ground nut oil showed large variations from sample to sample, most samples exhibiting no selective absorption but others showing well-defined ergosterol bands. In all the cases in which the ergosterol bands were distinct, there was also a band, more or less well defined, at 3200 Å. It will be recalled that a similar band was found by Schlutz and Morse, Lewkowitsch, Heilbron, Kamm, and Morton, and others in various animal oils such as whale oil, cod-liver oil, wool fat, and such. The authors say of it, "There is no definite evidence of its connection with vitamin chemistry. Experiments are now in progress designed to elucidate the nature of the compound corresponding to this band." These results are interesting in view of the more recent general acceptance of a correlation between absorption near 3200 Å and vitamin A, as discussed below.

With regard to olive oil they disagree with Lewkowitsch (45) who, it will be recalled, found a band at 2750 Å. They state, "In view of the frequent contamination of olive oil with cotton seed oil, and also since samples known to be free from adulterants examined by us did not show any band, the observations of Lewkowitsch should be accepted with reserve."

WOODROW'S RESULTS FOR COD-LIVER OIL

Woodrow (48; 1928) examined the absorption spectrum of cod-liver oil (A1) using an absorbing layer 0.04 mm. thick. His curve, reproduced in Figure 25, shows indications of the three absorption bands of ergosterol at 2700, 2800, and 2900 Å.

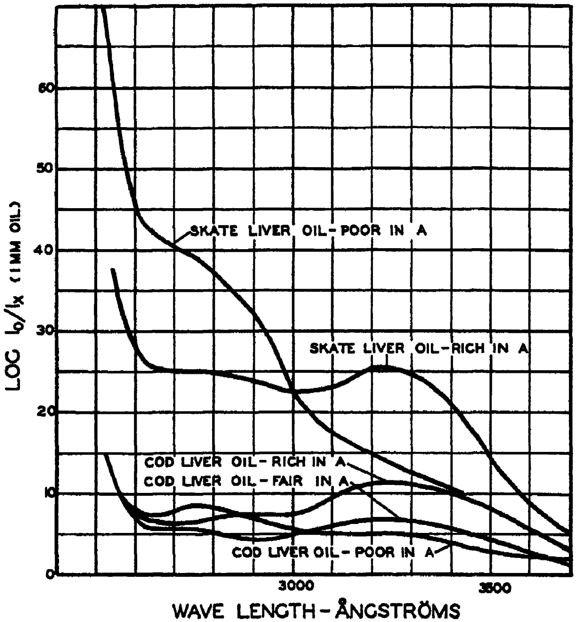
ULTRA-VIOLET ABSORPTION IN RELATION TO VITAMIN A

Since Woodrow's work, considerable interest has been exhibited in the absorption of oils from the standpoint of their vitamin A content. It was necessary to consider this work because of the presence of ergosterol in various oils examined.

The material is presented here for the sake of completeness, with apologies for wandering so far from our story.

Takahashi, Nakamiya, Kawakami, and Kitasato (49; 1925) ascribed absorption at 3200 Å found in concentrates from the non-saponifiable fractions of liver oils ("biosterin") to vitamin A.

Peacock (50; 1926) showed by rough spectrum photographs a decreased absorption in white-light-irradiated cod-liver oil which had lost its vitamin A potency as measured by color tests. Normal cod-liver oil had a transmission "limit" of 4100 Å., and irradiated oil of 3900 Å. (for the spectrum photographs, refer to the original paper).



Poor skate liver oil, 1 per cent solution, 2 mm cell, rich skate liver oil, 1 per cent solution, 4 mm. cell, cod-liver oils, 1 per cent solutions, 1 cm cell, solvents not stated (GGccI, II). See footnote page 203.

Fig. 26—Correlation of Vitamin A Content of Oils with Absorption at 3200-3280 Å. [Morton and Heilbron (51; 1928)].

Morton and Heilbron (51; 1928) early adopted the viewpoint that vitamin A should show selective absorption in the nearer ultra-violet, because it had been demonstrated by such workers as Peacock (50) that vitamin A can be destroyed by sunlight, the light from an incandescent lamp, and especially light from a quartz mercury arc.

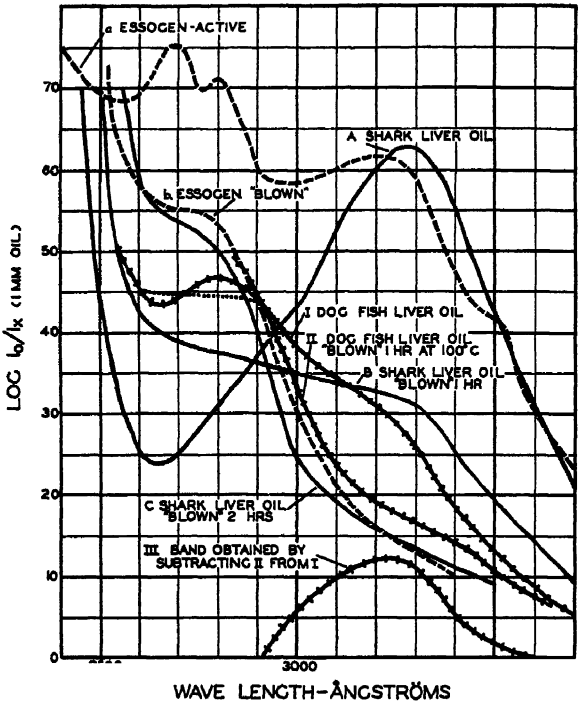
ASSOCIATION OF 3200-3280 Å. BAND WITH VITAMIN A

Since cod-liver oil is known to be rich in vitamin A, and since Schlutz and Morse (3), Heilbron, Kamm, and Morton (46), and Woodrow (48) had all found a maximum at about 3200-3280 Å. in cod-liver oil, they considered it possible that this band might be characteristic of vitamin A. It will be recalled that Lewkowitsch (45) also found such a peak for whale oil.

Morton and Heilbron's experiments indicated that most medicinal cod-liver oils show but a marked inflection in the 3200-3280 Å. region, but that unrefined oil from fresh livers shows a well-defined absorption band with a maximum at 3285 Å.

Examination of several samples of oil showed that the intensity of this absorption band varied directly with the depth of blue color obtained with the antimony trichloride color test for vitamin A. This correlation was found for other oils as well as cod-liver oil (Figure 26.) A commercial preparation "essogen," which gives a marked reaction with antimony trichloride, showed a clear band at 3280 Å. Unsaponifiable vitamin A concentrates, extracted from cod-liver oil and sheep-liver fat by Professor Drummond, and free from sterols precipitable by digitonin, showed the band strongly, the intensity being 200-300 times that in typical cod-liver oil for the cod-liver oil extract, and 800-1,000 times for the sheep-liver extract. Professor Drummond gave respective estimates of vitamin A potency of 500 and 775, based on color tests.

Dogfish oil, known to be rich in vitamin A and giving a strong color test, did not show a strong 3280 Å. band. The band was believed to be masked by the absorption of other materials, however, because of the high selective absorption near 2800 Å. "Blowing" the oil with oxygen for one hour at 100° C., to destroy the vitamin A content, resulted in a loss of the blue color reaction and a decreased absorption in the 3280 Å. region. When the absorption curve of the "blown" oil was subtracted from that of the original oil, a band with a maximum at about 3200 Å. was obtained, supposedly correlated with the vitamin A content destroyed by oxidation (Figure 27). The necessity of taking into consideration such masking of the 3280 Å. absorption is pointed out.



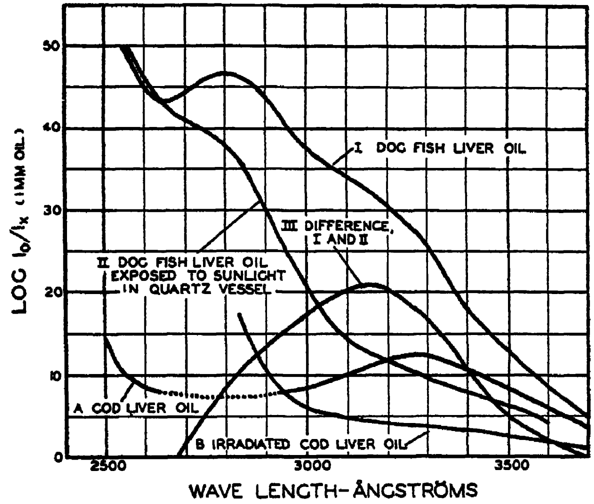
Curves a, b, A, B, and C 1 per cent solutions, 2 mm. cells, curves I and II, 1 per cent solutions, 3 mm cells; solvents not stated (GGeol). See footnote page 203.

Fig. 27—Absorption of Various Vitamin A-Containing Substances Before and After "Blowing" [Morton and Heilbron (51; 1928)].

The fact that Morton, Heilbron, and Kamm (46) had previously observed a band at 3180-3190 Å. in arachis oils seemed troublesome because vegetable oils are often considered free from vitamin A, but they cite the work of Bacharach (52; 1928), who found the vitamin in soya bean oil, as an indication that such materials may not be entirely devoid of the vitamin. The location of the band at 3180 Å. was suggested to be due to solvent shift, arachis oil being examined in its natural state while the other oils were in alcohol solutions.

EFFECT OF "BLOWING" OR IRRADIATING OILS

Tests of "blown" cod, dog-fish, and shark-liver oils, and of blown essogen showed that when the aeration resulted in a reduction of the color reaction (oxidation being known to destroy vitamin A) there was a proportionate reduction in the 3280 Å. absorption band (Figure 27). Irradiation of cod-liver oil (likewise known to decrease the vitamin A potency) also reduced the intensity of the 3280 Å. band, and the intensity of the blue color reaction (Figure 28). Samples irradiated through a silvered quartz filter (which transmits a band of the ultra-violet in the 3280 Å. region) produced the same results. This latter experiment was evidently undertaken with the view that chemical change



Curves I and II, 1 per cent solutions, 3 mm. cells; curves A and B, 1 per cent solutions, 1 cm. cells; solvents not stated (GGccI, II). See footnote page 203.

Fig. 28—Absorption of Liver Oils Before and After Irradiation [Morton and Heilbron (51; 1928)].

takes place only in regions of selective absorption, the decreased color reaction obtained indicating from that viewpoint an association of the 3280 Å. absorption band with the substance responsible for the color reaction.

In some of the oils poor in vitamin A or in which the vitamin content had been destroyed, a band was found in the 2750-2850 Å. region. The possibility of this band being associated with destruction products of vitamin A is suggested. A similar band was found in cod-liver oil concentrates. An inflection at about 2300 Å. was also observed in many oils, but no specific significance was attached to it.

Table V is a copy of their table of results, of interest not only from the vitamin A standpoint but because of the ergosterol bands found in the various oils.

ROSENHEIM AND WEBSTER CRITICIZE THE ASSOCIATION OF VITAMIN A WITH 3280 Å. ABSORPTION

Rosenheim and Webster (53; 1929) criticized the association of vitamin A with a 3280 Å. absorption band by Morton and Heilbron and by Takahashi and his coworkers on the grounds that they found dehydroergosterol to have an intense band in exactly the same region. The absorption curve of this material had previously been obtained by Windaus and Linsert (54; 1928). Feeding tests showed dehydroergosterol not to have growth-promoting properties, and tests with $AsCl_3$ or $SbCl_3$ failed to show the blue color reaction. "It would seem therefore," they say, "that selective ultra-violet absorption at 3200-3280 Å., at any rate by itself, cannot be taken as a criterion of vitamin A."

This seems a rather weak argument in view of the fact that many substances of quite dissimilar properties frequently exhibit ultra-violet absorption in the same region, by consequence of which the finding of an inactive substance having absorption similar to that attributed to vitamin A would hardly invalidate such absorption for vitamin A unless it could be proven definitely that this inactive substance is the only one responsible for the absorption in the region in question by the materials investigated. Rosenheim and Webster do not offer such proof.

TABLE V

Ultra-Violet Absorption of Various Oils, According to Morton and Heilbron (51, 1928).

MATERIALS	BAND OR INFLEXION NEAR 230$\mu\mu$	BAND OR INFLEXION NEAR 270-290$\mu\mu$	BAND OR INFLEXION 320-328$\mu\mu$	UNITS OF ABSORPTION*	UNITS OF "BLUE"†
Marine animal oils of known origin:					
(1) *Hexanchus*	—	Faint inflexion	Absent	0	0
(2) *Spinax niger*	—	Definite	Absent	0	0
(3) *Scyllium canicula*	—	Faint	Absent	0	0
(4) *Scymnodon ringeris*	—	Faint	Absent	0	0
(5) *Raia clavata*	Inflexion	Definite	Doubtful	0	0
(6) *Raia batis*	Inflexion	Definite	Absent	0	0
(7) *Scymnorhinus lichia*	Inflexion at 240$\mu\mu$	Marked at 275$\mu\mu$	Absent	0	1.0 *circa*
Cod-liver oils from Prof. Hilditch:					
(1) Oil from fresh livers	—	Ergosterol bands	Good band	10.8	9.9
(2) Chilled and filtered oil	—	Masked if present	Good band	11.0	9.9 approx.
(3) Non-freezing oil	No inflexion	Masked if present	Good band	12.0	10.5
(4) Stearin-rich oil	—	Masked if present	Good band	10.0	10.5 approx.
Blown oils from fresh livers:					
4 hours at 43°	—	No change	No change	12	Not measured
4 hours at 80°	—	Definite inflexion	Less intense	6	Not measured
4 hours at 80°	—	Definite	Less intense	<2.5	Not measured

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*The arbitrary unit of absorption here used may be defined as follows: when log I_0/I (intensities of incident and emergent light respectively) represents the extinction E, 1 unit of absorption for a 1 per cent solution and a 1 cm. cell corresponds with a persistence of $E = 0.04$ for the 328$\mu\mu$ band.

†1 unit is given by log $I_0/I = 0.275$ at 608$\mu\mu$ using 2 cm. cells, 0.3 cc. for 10 per cent cod-liver oil in chloroform plus 3 cc. of 30 per cent antimony trichloride in the same solvent. This unit stands in close relationship with Lovibond units.

—indicates that definite results were not obtained.

TABLE V (*continued*)

MATERIALS	BAND OR INFLEXION NEAR 230$\mu\mu$	BAND OR INFLEXION NEAR 270-290$\mu\mu$	BAND INFLEXION 320-328$\mu\mu$	UNITS ABSORPTION	UNITS OF "BLUE"†
Irradiated oil:					
Fresh liver oil irradiated for 4 days	—	Definite	Absent	0	0
Cod-liver oils of high medicinal quality from Prof. Drummond:					
"G" Scotch oil from Aberdeen	—	Ergosterol	Good band	11 5	10 0
"K" Lofoten oil	—	Inflexion 275	Smaller band	5 8	6.6
"L 4" Lofoten oil	—	Inflexion 275	Clear inflexion	3 2	3 3
Essogen (Lever Bros.)	—	Ergosterol definite	Clear band	<112	Not measured
Essogen blown with oxygen for 1 hour at 100°	—	Clear inflexion	Absent	0	0
Ostelin	Inflexion	Clear inflexion	Doubtful	?	?
Shark-liver oils:					
Sample (1)	Inflexion	Ergosterol (?)	Inflexion	?	?
Sample (2)	—	Inflexion	Inflexion	<12 5	12
Sample (3)	—	Band at 280$\mu\mu$	Very weak	<12 5	14.5
Sample (4)	Good inflexion	Masked if present	Good band	72	80
Sample (5)	Good inflexion	Masked if present	Good band	94	90
Irradiated shark oil (4) (silvered quartz test-tube)	—	Inflexion	Much weakened	Not measured	
Blown shark oil (5):					
(1) oxygen for 1 hour at 100°	—	Inflexion	Weaker	Doubtful	13 } doubtful values
(2) oxygen for 2 hours at 100°	—	Marked inflexion at 275$\mu\mu$	Much weaker	Doubtful	11
Porbeagle shark-liver oil	—	Small inflexion	Very doubtful	0	0

Skate-liver oils:					
Sample (1)	—	Clear inflexion at 275μμ	Poor	?	<3 5
Sample (2)	—	Marked inflexion	Good band	Order 25	19
Dogfish-liver oils:					
Sample (1)	—	Band at 280μμ	Inflexion (See Fig. 1)	38	28
Sample (1) (blown with oxygen 1 hour at 100°)	—	Band at 280μμ	No inflexion	0	?
Sample (1) (exposed to sunlight in a quartz vessel)	—	Faint inflexion	Weak if at all	?	8 5
Whale oil	—	Inflexion	Faint	?	?
Wool fat	—	Inflexion	Faint band	?	Positive
Spent cod-liver oil from the Zucher process	—	Inflexion	Small band	Order 2	Not measured
"Un-sap." from cod-liver oil after removal of all sterols precipitable by digitonin (from Prof. Drummond)	No inflexion	Masked if present	Good band	600–1000	1650
"Un-sap." from sheep liver similarly prepared (from Prof. Drummond)	No inflexion	Masked if present	Good band	2500–3000	2580

FURTHER CORRELATION BETWEEN VITAMIN A AND 3280 A. ABSORPTION

Drummond and Morton (55; 1929) made a more extensive study of the coördination between absorption at 3280 Å., the colorimetric test, and the biological test for vitamin A.

A number of samples of oils were used and tested before and after ageing under various conditions. From the absorption curves, the absorption of oils in which the vitamin A content had been destroyed by various means was subtracted, the resulting differences in extinction being used as estimates of the vitamin A content. Table VI shows the degree of correlation obtained between the spectrographic, colorimetric, and biological tests. Detailed data and curves are presented, which it is not essential to review here.

TABLE VI

Comparison of Biological and Physical Measurements of the Relative Vitamin A Value of Six Cod-Liver Oils, according to Drummond and Morton (55; 1929)

OIL	BIOLOGICAL METHOD	COLORIMETRIC METHOD		ULTRA-VIOLET BAND
		TINTOMETER	SPECTROMETER	
C	2	2 5	2 65	2 65
F	5	3 3	3 3	3 25
G	>2	3 0	3 15	3 25
J	2	1 7	1 7	1 5
K	1 5	1 8	1 77	1.7
L	1	1 0	1 0	1 0

MORTON, HEILBRON, AND SPRING ANSWER THE CRITICISM OF ROSENHEIM AND WEBSTER

Morton, Heilbron, and Spring (56; 1930) replied to Rosenheim and Webster's (53) criticism, pointing out that the correlation between 3280 Å. absorption and growth-promoting properties had been claimed only for growth-promoting factors found in liver oils and not for such factors of vegetable origin, which might be quite different; and that it was not claimed that *any* substance absorbing near 3000-3500 Å. is vitamin A, since many substances absorb in that region. They further state that

Rosenheim and Webster, in basing their conclusions on superficial similarities in absorption, have neglected both fine structure and degree of absorption. Upon re-determining the absorption of dehydroergosterol they found three peaks, not present in the spectra of their oils (their curves of dehydroergosterol will be reproduced in the discussion of the chemical nature of vitamin D), and the values of absorption that they obtained were less than those of the vitamin A in liver oils if the 3280 Å. absorption in these oils could be attributed to their vitamin A content.

RELATION OF CAROTENE TO VITAMIN A

Absorption spectra have played an important part in the investigation of the relation of carotene to vitamin A. The observation that yellow foodstuffs frequently exhibit strong vitamin A properties naturally led to an assumption of the identity of carotene and vitamin A. Euler, Euler, and Hellström (57; 1928) stated that the feeding of small doses of carotene (0.01 to 0.05 mg.) to rats on a vitamin A deficient diet restored growth to the animals. They concluded that vitamin A is carotene or a similar substance.

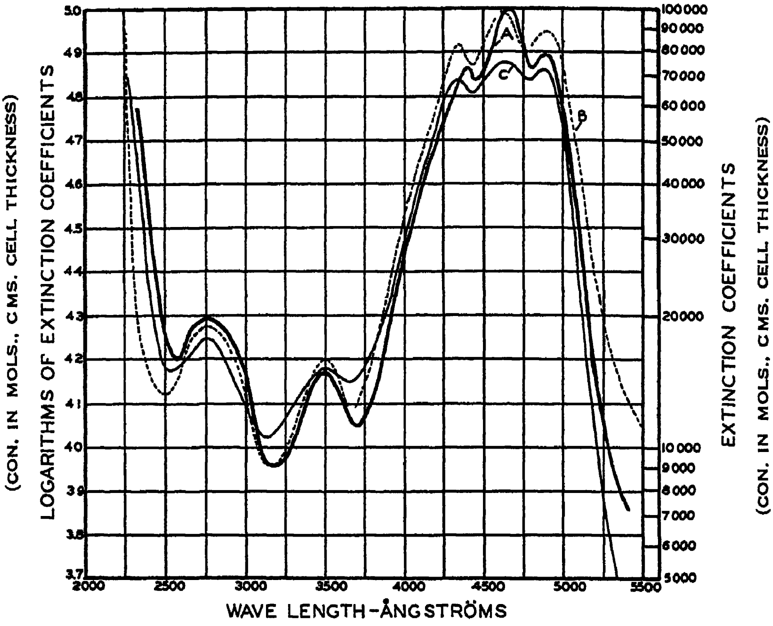
FAILURE OF DULIERE, MORTON, AND DRUMMOND TO OBTAIN GROWTH-PROMOTION WITH CAROTENE

Duliere, Morton, and Drummond (58; 1929) criticized the findings of Euler, Euler, and Hellström (57) because they failed to obtain growth-promotion with highly-purified carotene (m.p. 184.9° as compared with 167.5° to 178.0° obtained by other workers) in doses comparable to those used by Euler et al., because they found that carotene did not have an ultra-violet absorption band at 3200-3300 Å. "associated with the vitamin," and because the antimony trichloride color reaction with carotene gave a maximum at 5900 Å. as compared with 6080-6120 Å. for vitamin A concentrates. They concluded that the results of Euler et al. were due to contamination of their carotene with vitamin A.

A more detailed account of the findings of Duliere, Morton, and Drummond was published later (59; 1929), in which they give spectrograms (Adf6) of carotene of various melting points[1] (Fig. 29). They reaffirmed their conclusions stated above.

CONFIRMATION OF GROWTH-PROMOTING ACTION OF CAROTENE BY OTHER WORKERS

In the meantime, however, the biological activity of carotene had been confirmed by Moore (65, 66; 1929), Collison, Hume, Smedley-MacLean, and Smith (67; 1929), Kawakami and Kimm (68; 1929), and Euler, Euler, and Karrar (69; 1929).



Chloroform solutions, concentrations and thicknesses not stated (EE). See footnote page 203.

Fig. 29—Absorption of Carotene [Duliere, Morton, and Drummond (59; 1929)].

Curve *A*, m.p. 185°; curve *B*, m.p. 182°; curve *C*, m.p. 171°.

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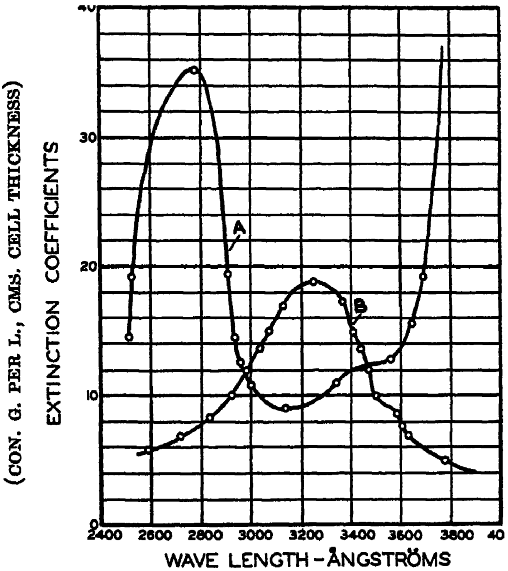
[1] References are given in their article to the following papers discussing the absorption of carotene in the visible region: Kohl (60; 1902), Willstätter and Stoll (61; 1913), Tschirch (62; 1904), and Pummerer and Rebmann (63; 1928). Those interested in carotene will also find a recent paper by Bilger (64; 1931), and a still more recent one by McNicholas (82; 1931) of value. The former gives spectrum photographs and the latter absorption curves of carotene and other pigments both in the visible and ultra-violet.

Hume and Smedley-MacLean (70; 1930) suggested, in view of experiments conducted by them, that the negative results obtained by Duliere, Morton, and Drummond (58, 59) in feeding carotene were due to the use of ethyl oleate as a solvent for the carotene by those authors.

EVIDENCE THAT THE ACTIVITY OF CAROTENE IS NOT DUE TO CONTAMINATION WITH VITAMIN A

Having at hand these results, Capper (71, 1930) thought it advisable to compare the biological activity of carotene with its absorption at 3280 Å. in order to see whether the activity of carotene found by various workers could be attributed to traces of the vitamin A associated with absorption at 3280 Å.



Curve A, 0.006 per cent in dry ether, 1 cm. cell; curve B, 0.005 per cent in dry ether, 1 cm. cell (GGccII). See footnote page 203.

Fig. 30—Absorption of Carotene and Cod-Liver Oil Concentrate [Capper (71; 1930)].

Curve *A*, carotene; curve *B*, cod-liver oil concentrate.

Figure 30 shows his results in comparing the absorption of carotene and cod-liver oil concentrate, both in the same concentrations. The absorption of carotene is less in the 3280 Å.

region while its biological activity was found to be greater (minimum daily dose of carotene, 0.002 mg.; of cod-liver oil concentrate, 0.01 mg.) from which it was deduced that the biological activity of carotene cannot be explained by contamination with a vitamin A characterized by 3280 Å. absorption.

Capper suggests: (1) that the association of 3280 Å. absorption with vitamin A in cod-liver oil may be fortuitous; (2) that vitamin A may exist in two forms, only one of which shows strong absorption at 3280 Å.; and (3) that vitamin A may be synthesized in vivo from carotene as suggested by Moore (72; 1929) or from an impurity in carotene.

EVIDENCE THAT CAROTENE IS CHANGED TO VITAMIN A IN VIVO

Moore (72, 1929; 73, 1930; 74, 1930) described experiments indicating that carotene is changed to vitamin A in the livers of animals. Liver oils from rats on a vitamin A-free diet gave negative color tests with antimony trichloride and failed to show an absorption band at 3280 Å. (the absorption measurements being carried out by Capper). When the rats were fed carotene (m.p. 178°), carrots, or red palm oil, they lost their vitamin A deficiency symptoms, and their liver oils showed an intense band at 6100-6300 Å. with antimony trichloride, an absorption band at 3280 Å., a marked biological activity, and a lack of the strong yellow color which would be expected if carotene were stored in the livers as such.

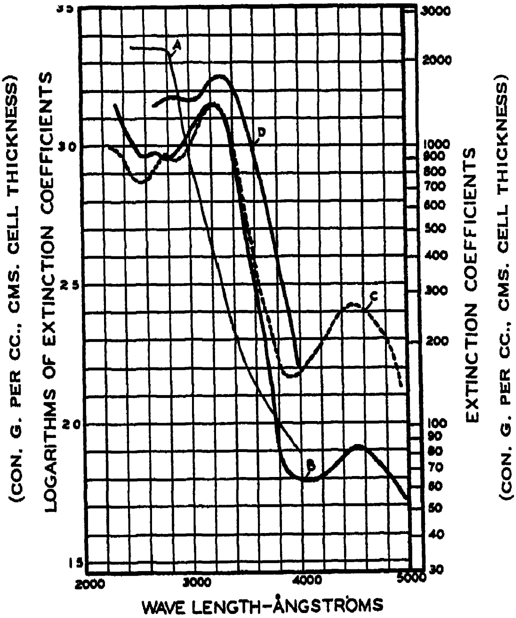
The ultra-violet spectra of the liver oils used in Moore's experiments are described by Capper (75; 1930). He found no band at 3250 Å.[1] in liver oils from rats on a vitamin A-free diet, but did find this band in liver oils from rats to the diet of which carotene or carotene-containing substances had been added. No band at 3250 Å. was found in red palm oil, administered as a carotene-containing substance. His results are reproduced in Fig. 31 and Table VII.

---

[1] His previous paper and the results of other workers place the absorption maximum characteristic of vitamin A in liver oils at 3280 Å.

DRUMMOND, AHMAD, AND MORTON ACKNOWLEDGE THE ERROR OF THE NEGATIVE EXPERIMENTS OF DULIERE, MORTON, AND DRUMMOND

Finally, Drummond, Ahmad, and Morton (76; 1930) acknowledged that the failure of Duliere, Morton, and Drummond



Curves A and D, chloroform solutions; curves B and C, ether solutions; concentrations and cell thicknesses not stated (EE). See footnote page 203.

Fig. 31—Absorption of Liver Oils from Rats Fed Carotene [Capper (75; 1930)].

Curve *A*, liver oil of rat No. 10 (see table VII) fed vitamin A-free diet; curve *B*, liver oil of rat fed carotene; curve *C*, liver oil of rat fed red palm oil; curve *D*, liver oil of rat fed carrots.

(59) to obtain growth-promotion with carotene was due to the rapid oxidation of the carotene in the ethyl oleate used as a solvent. Using other solvents, they were able to confirm the marked growth-promoting properties of carotene, found by

other workers. They also confirmed findings of Euler and his colleagues regarding the growth-promoting activity of dihydro-α-crocetin. Absorption spectra of α-crocetin and dihydro-α-crocetin are given (Figure 32).

Palm oil, previously said to be growth promoting, was found to be active. Its absorption spectrum (Fig. 33) "revealed that the only selective absorption was that of carotene and that the concentration was between 0.2 per cent and 0.3 per cent." The different positions of the maxima in palm oil and its unsaponifiable fraction is attributed to the different solvents used. "The values in each case agree well with those of carotene in the same solvents."

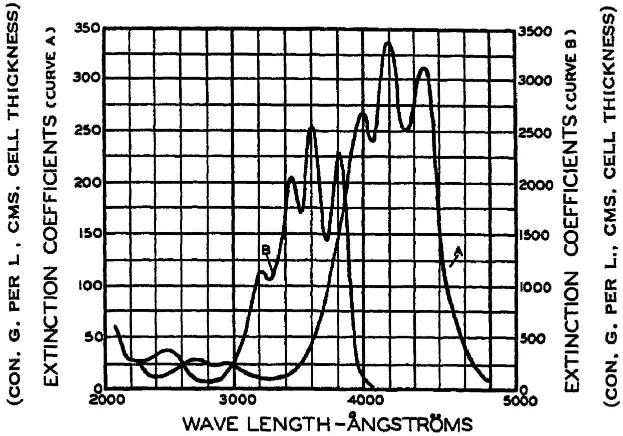
TABLE VII

Ultra-Violet Absorption of Liver Oils as Determined by Capper (75; 1930).

RAT No.	DIET	SOLVENT	BAND AT 3250 Å.	BAND OR INFLECTION IN REGION OF 2800 Å.
5	Vitamin A-free	Ether	No band	Inflection
10	Vitamin A-free	Ether	No band	Inflection
8	Vitamin A-free	Chloroform	No band	Inflection
9	Vitamin A-free	Chloroform	No band	Inflection
16	Vitamin A-free + 0.75 mg. per day carotene, m.p. 178°	Ether	Good band	Shallow band
17		Chloroform	Good band	Shallow band
20	Vitamin A-free + red palm oil (1.5 g.)	Ether	Good band	Shallow band
19		Chloroform	Good band	Inflection
22	Vitamin A-free + fresh carrots ad. lib.	Chloroform	Good band	Shallow band

Spectrographic examination of the liver oils of rats fed carotene showed the presence of the 3280 Å. "vitamin A" band, in support of the findings of other workers that carotene is transformed to vitamin A in the liver (Fig. 34).

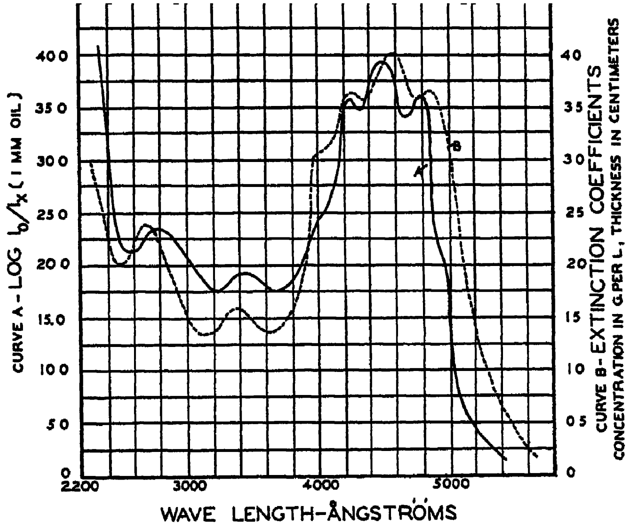
The following very positive statement is made in the dis-



Curve A, 0 008 per cent in 1 per cent caustic soda solution, 5 mm cell, curve B, 0.002 per cent in 1 per cent caustic soda solution, 2 mm. cell (GGccl). See footnote page 203.

Fig. 32—Absorption of α-Crocetin and Dihydro-α-Crocetin [Drummond, Ahmad, and Morton (76; 1930)].

Curve *A*, α-crocetin; curve *B*, dihydro-α-crocetin.



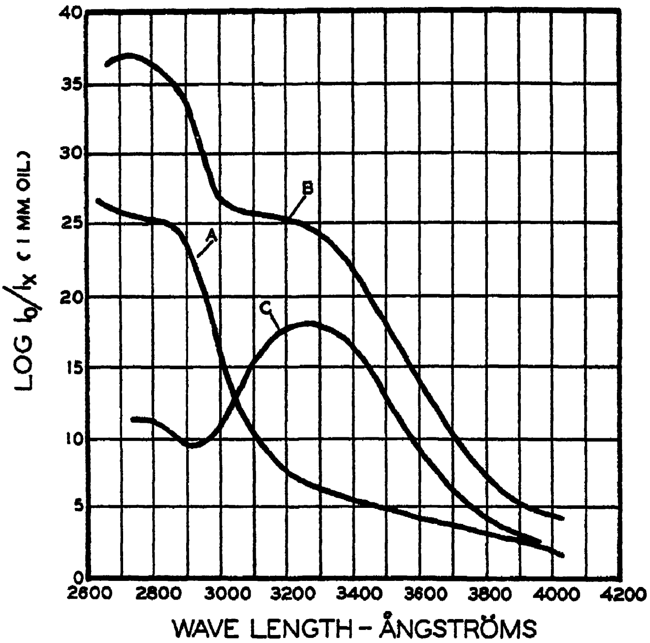
Curve A, 1 per cent in alcohol, 4 mm. cell; curve B, 0 122 per cent in chloroform, 3 mm. cell (GGccl). See footnote page 203.

Fig. 33—Absorption of Palm Oil (curve *A*) and Non-Saponifiable Fraction from Palm Oil (curve *B*) [Drummond, Ahmad, and Morton (76; 1930)].

cussion: "There is, of course, no question of the identity of vitamin A with carotene. The spectroscopic evidence presented by us previously (59) dismisses this possibility once and for all."

THE ANTIMONY TRICHLORIDE REACTION AND ULTRA-VIOLET ABSORPTION

Morton, Heilbron, and Thompson (77; 1930) reported some data regarding the antimony trichloride reaction and the absorption at 3280 Å. in various vitamin A-containing materials.



0 97 per cent solutions in alcohol, 4 mm. cell (GGccI) See footnote page 203.

Fig. 34—Absorption of Liver Oils of Rats Fed Carotene [Drummond, Ahmad, and Morton (76; 1930)].

Curve *A*, low carotene diet; curve *B*, high carotene diet; curve *C*, curve *B* minus curve *A*.

Liver oils are said to show a maximum at 6060 Å. with antimony trichloride and at times a secondary maximum at 5650-5850 Å. which at times "even predominates," the strong secondary maximum being accompanied by increased absorption at 3280 Å.

Concentrates gave a primary color reaction band at 6240 Å. and secondary ones at 5820-5930 Å., 6430 Å., and 6970 Å. Their richest concentrate "exhibited strong parallelism" between the 3280 Å. ultra-violet band and the 6240 Å. color-reaction band. Dilution of concentrates with seal oil shifted the 6240 Å. color reaction band to 6060 Å., "the normal location for cod-liver oil." A fish-liver oil concentrate reduced almost to dryness with alcoholic sodium ethoxide at 180° and extracted showed a disappearance of the 3280 Å. absorption, and a much reduced color reaction, with a single maximum at 5830 Å. New bands appeared in the ultra-violet spectrum, however, at 3940, 3730, 3480, 3300, 3170, 3010, 2900, 2820, 2710, and 2600 Å., these being "associated with an acid," of which the chemical properties were said to be under investigation.

ULTRA-VIOLET ABSORPTION OF BUTTER IN RELATION TO VITAMIN A

Morton and Heilbron (78, 79; 1930) studied the ultra-violet absorption of the non-saponifiable fraction of butter with regard to its content of carotene and of the substance in liver oils having absorption at 3280 Å. They state that carotene in chloroform has absorption bands at 4920, 4620, 4360, 3480, and 2790 Å.; while in alcohol the visible bands are at 4800, 4510, and 4270 Å. The strong visible and comparatively weak ultra-violet absorption of carotene is contrasted with the weak visible and strong ultra-violet absorption of the unsaponifiable extracts of liver oils.

Carotene is said to give a blue color reaction with antimony trichloride with a maximum at 5910 Å., and the non-saponifiable material from liver oils a similar reaction with a maximum at 6240 Å.

Extracts from New Zealand butter and from Danish butter showed both the carotene absorption bands and an inflection at 3280 Å. Both samples had a strong maximum near 2720 Å., not identified with any particular substance. The 2720 Å. maximum was stronger in the Danish butter fraction, and it is suggested that the substance responsible for it may account for a considerable part of the 3280 Å. absorption in this sample.

Estimates of the quantity of carotene in the samples were made by comparing the intensity of the carotene absorption maxima in the butter extracts with the same maxima in spectra of pure carotene and by comparing the 5910 Å color reaction obtained with the butter extracts with the reaction obtained with pure carotene. By similarly comparing the 3280 Å. absorption with that of a sample of cod-liver oil and the 6240 Å. color reaction with that obtained by using cod-liver oil, estimates of the content of cod-liver oil vitamin A were made. The results were as follows:

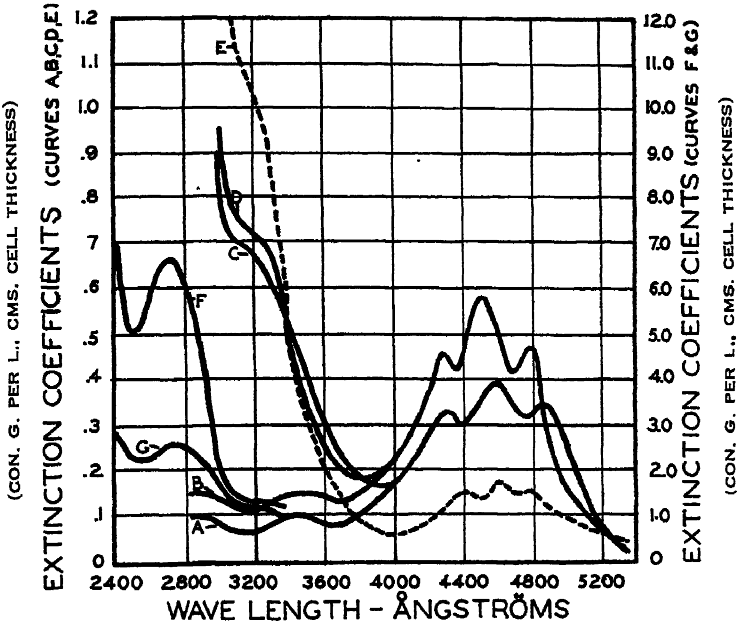
	SPECTROGRAPHIC ESTIMATE	COLOR TEST ESTIMATE
New Zealand Butter Extract:		
Carotene Content	0.5%	2.0%
Maximum Liver Oil A Content	6x good c. l. oil	5x good c. l. oil
Danish Butter Extract:		
Carotene Content	0.1–0.15%	Negative
Maximum Liver Oil A Content.	6x good c. l. oil	2.45x good c. l. oil

FURTHER DATA REGARDING THE ABSORPTION OF LIVER OILS

Morton, Heilbron, and Thompson (80; 1931) published further data in 1931 regarding liver oils and oil concentrates. They found the many cod-liver oils investigated to be characterized by a single, well-defined maximum at 3200-3300 Å., with some indication of a break at 3000-3100 Å. The 3280 Å absorption agreed well with the antimony trichloride color reaction, as pointed out in their previous communications. A few oils of low vitamin A potency showed faint, narrow bands between 3000 and 3500 Å. They decided that the "true vitamin A ultraviolet band is continuous and free from fine structure."

The extinction coefficient (log I_x/I_o) at 3280 Å. for a 1 cm. thickness of 1 per cent solution in alcohol varied for "genuine refined" cod-liver oils from 0.2 to 2.0, most of them being in the range 0.5 to 1.6. Carr and Price vitamin A units may be obtained approximately by multiplying these figures by 12, they state. In terms of 1 mm. layers of pure oil, which we have used as a basis of plotting and comparison herein, the extinction limits would be 2.0 to 20.0 for all samples, and in the range 5.0 to 16.0 for the majority. The general absorption due to other factors must be subtracted from the total absorption at 3280 Å.

in order to obtain the true "vitamin A" absorption. A satisfactory correction is from 0.2 to 0.3 (our units, 2.0 to 3.0) for weakly colored oils, they say, and somewhat greater for highly colored oils. In some cases of fish-liver oils other than cod-liver oils the absorption due to vitamin A appeared to be masked by strong absorption in this region due to unknown factors, as in the case of dogfish-liver oil discussed on page 213.



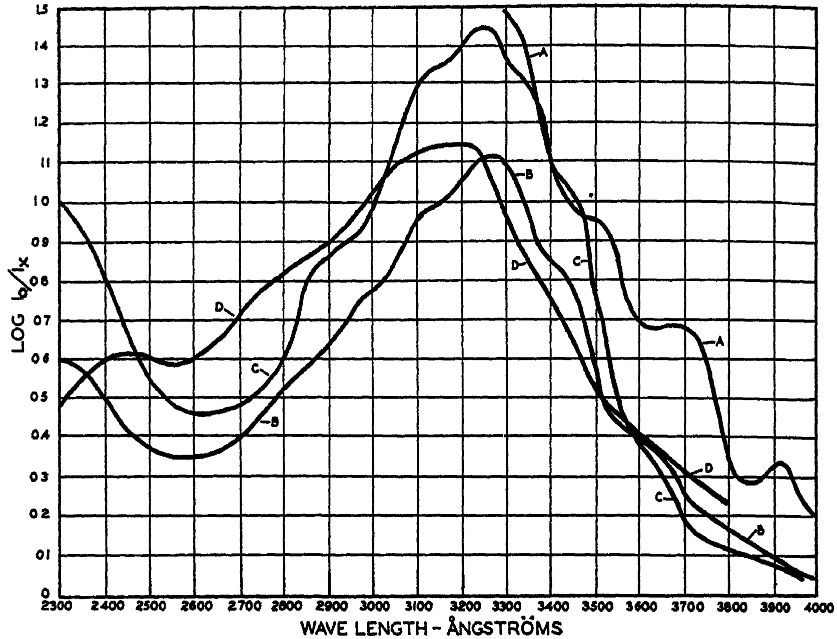
Curves A and B, concentration, solvent, and cell thickness not stated; curve C, 0.78 per cent in chloroform, 2 mm. cell; curve D, 0.80 per cent in alcohol, 2 mm. cell; curve E, 0.4 per cent in alcohol, 2 mm. cell; curve F, 0.107 per cent in alcohol, 2 mm. cell; curve G, 0.10 per cent in alcohol, 2 mm. cell (GGccI). See footnote page 203.

Fig. 35—Absorption of Non-Saponifiable Extracts from Butters [Morton and Heilbron (78; 1930)].

Curve *A*, carotene; curve *B*, carotene; curve *C*, New Zealand butter extract in chloroform; curve *D*, New Zealand butter extract in alcohol; curve *E*, Danish butter extract in alcohol; curve *F*, Danish butter extract in alcohol; curve *G*, New Zealand butter extract in alcohol.

ABSORPTION IN THE ERGOSTEROL REGION

Nearly all the oils they investigated showed selective absorption in the 2600-2950 Å. region, resolution into peaks being poor in most cases, but they did not find it possible to correlate absorption at 2800 Å. with the vitamin D content, or absorption in this region with the ergosterol content (compare their previous work, page 209).



Curve A, heptane solution, concentration and cell thickness not stated; curves B and C, 0.46 per cent in alcohol, 2 mm. cell; curve D, 0.035 per cent in alcohol, 1 mm. cell (GGccI). See footnote page 203.

Fig. 36—Absorption of Vitamin A Concentrates Showing Fine Structure [Morton, Heilbron, and Thompson (80; 1931)].

Curve *A*, sheep liver concentrate left in contact with air and extracted with heptane; curve *B*, liver oil concentrate 200 times as potent as cod-liver oil; curve *C*, extract from *B*; curve *D*, concentrate 1000 times as potent as cod-liver oil.

Liver oil concentrates showed correlation between the 3280 Å. absorption band and the blue color reaction. By using concentrates 200 times as potent as cod-liver oil in vitamin A, they obtained evidence of fine structure in the 3280 Å. band (Figure 36), but a concentrate 1,000 times as potent showed less indications of fine structure, from which they concluded that the apparent breaks in the curve must be due to substances other than vitamin A.

To test this view, they used chemical methods to remove the 3280 Å. band associated with vitamin A. Such treatment of fish-liver oils resulted in absorption peaks at 3940, 3700, 3500, and 3340 Å.

FINE STRUCTURE DUE TO ACIDS

Repeated treatments of cod-liver oil concentrate with sodium ethoxide until the characteristic vitamin A color reaction no longer was obtained was followed by the appearance of many absorption bands (Figure 37). Spectrographic examination of the acids extracted from their treated material revealed the same bands, indicating that the acid content of the treated concentrate was responsible for the selective absorption found in the latter.

Repeating treatment of the concentrate at a higher temperature and with sodium amyloxide instead of sodium ethoxide so as to destroy more effectively the vitamin A content, and extracting the fatty acids, yielded a product also showing similar spectrum bands, but of different intensity, from which the authors concluded that the acidic absorbing material was heterogeneous, consisting of at least three different selectively absorbing acids. The bands at 2600, 2710, and 2820 Å. were associated with one substance, those at 3010 and 3170 Å. with another, and those on the long wave side of 3170 Å. with a third, selective absorption shorter than 2600 Å. "remaining to be accounted for."

Acids obtained by the ordinary saponification of cod-liver oil showed the same type of spectra. On the basis of the intensity of absorption of the acids and the lack of appearance of these bands in ordinary cod-liver oil spectra they conclude that these acids are not present as simple glycerides in the oil itself but are products of saponification or alkali treatment.[1]

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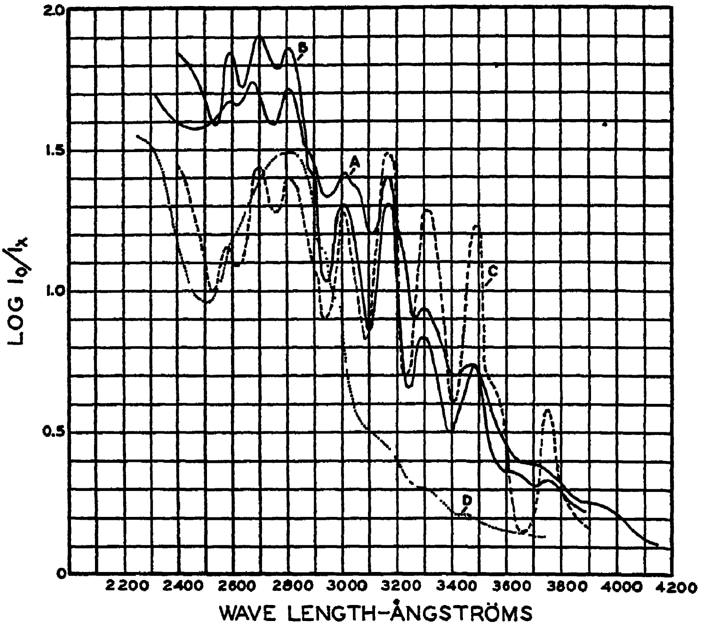
[1] The differences in extinction between pronounced peaks and adjacent hollows found in the acids were about 0.5 for 0.02 % alcohol solutions in 1 cm. cells. The extinction coefficient at 3280 Å. for a 1% solution of a typical oil

Gillam, Heilbron, Hilditch, and Morton (81; 1931) made a further study of the absorption of acids obtained from cod-liver oil and other sources. There seemed to be a difference between



Curve A, 2 per cent, 2 mm. cell, solvent not stated; curve B, 0.02 per cent in alcohol, 0.5 cm. cell; curve C, 0.02 per cent in alcohol, 1.0 cm. cell curve D, 0.12 per cent in alcohol, 2 mm. cell (GGeeI, II). See footnote page 203.

Fig. 37—Absorption of Acids from Liver Oil Concentrates [Morton, Heilbron, and Thompson (80; 1931)].

Curve *A*, crude hexane extract from liver oil concentrate after sodium ethoxide treatment; curve *B*, acid fraction obtained from concentrate treated with sodium ethoxide; curve *C*, acid fraction obtained from concentrate treated with sodium amylate; curve *D*, acid fraction obtained from rich concentrate treated with sodium amylate.

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in a 1 cm. cell was about 1.3. According to their data the weight of the acids obtained by sodium ethoxide treatment and ether extraction was approximately 0.06% of the original cod-liver oil. Since 0.06% of 1% is 0.0006%, the acids present in the 1% cod-liver oil solution should have shown an absorption equivalent to a 0.0006% solution of the acids in alcohol. Since 0 02% solutions of the acids showed differences in extinction between peaks and hollows of 0.5, one would expect a 0.0006% solution to show differences of the absorption of acids obtained from oils containing vitamin A and those obtained from oils not containing vitamin A. A number of curves are given, for which one should refer to the original article.

CONCLUSIONS

The foregoing work seems to indicate that at the present stage of development ultra-violet absorption spectra frequently fail to show the presence of ergosterol and vitamin D in oils in amounts sufficient to be biologically important. Only when oils are rich in ergosterol are the bands evident with certainty, according to the work reviewed.

Qualitatively, the correlation between an absorption band with a maximum at about 3280 Å. and the presence of vitamin A seems fairly well established. Semi-quantitative agreement has been obtained, but a high degree of quantitative accuracy is necessarily hindered by the presence of an indeterminate amount of absorption due to other, unknown factors.

The growth-promoting action of carotene seems well established, while its differentiation from vitamin A is supported by the widely different ultra-violet absorption spectra of carotene and vitamin A-containing substances.

Considerable evidence has been presented to the effect that carotene is changed to vitamin A in vivo. In particular the absorption spectra of liver oils from rats fed carotene bear this out.

The ultra-violet absorption spectra of numerous liver oils and vitamin A-containing substances have been described in the literature. The acid fractions of the oils have been found to have characteristic fine structure spectra.

At the present moment one might say that it is practically universally accepted that vitamin A is characterized by an absorption band with a maximum near 3200 Å., but that point of view will no doubt be challenged before the final chapter of this subject is written.

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about 0.015, provided Beer's law holds. This is only about 1% of the extinction they found for cod-liver oil at 3280 Å. in 1% solution, 1 cm. cells, and it seems questionable whether or not their methods were sufficiently accurate to detect such minor variations in absorption.

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THE ULTRA-VIOLET ABSORPTION SPECTRA OF CERTAIN VITAMIN B-CONTAINING PREPARATIONS[1]

By FRANCIS F. HEYROTH AND JOHN R. LOOFBOUROW

SUMMARY

The absorption spectra of two vitamin B concentrates supplied by Dr. Atherton Seidell, and of one prepared by the authors, have been determined and have been found to resemble those of purines and pyrimidines investigated by the authors and previously reported in the literature. The resemblance is somewhat greater to the spectra of certain purine and pyrimidine irradiation products, especially if the irradiation is carried out with ultra-violet from which the shorter waves have been excluded. It is concluded that there is evidence to show the presence of purine- or pyrimidine-containing compounds, or modifications thereof similar to those brought about by irradiation with ultra-violet, in vitamin B concentrates.

The similarity of the sources of the nucleic acids to the sources of vitamin B made it seem desirable to examine vitamin B concentrates spectrographically for the presence of the type of ultra-violet absorption which, according to our earlier work (1), characterizes the pyrimidine ring (whether free or a part of a purine ring or a nucleic acid derivative).

REVIEW OF THE EVIDENCE RELATING THE NUCLEIC ACIDS TO VITAMIN B

In the course of the many attempts which have been made to isolate vitamin B, attention has frequently been drawn to the pyrimidines and the purines.

In 1912, C. Funk (2) obtained from rice polishings and from yeast, small quantities of a free base, probably of the pyrimidine group, which he thought to cure polyneuritic pigeons in 0.02 to 0.04 g. doses.

Later, he presented evidence to show that various purines and pyrimidines, but not uric acid, kept polyneuritic pigeons alive for four, five, or more days after the onset of the disease, while without such additions to the polished-rice diet they rarely lived longer than twenty-four hours. Allantoin or hydantoin added to a diet of polished rice delayed the onset of the symptoms and lessened the loss of weight. In all, uric acid, adenine, guanine, hypoxanthine, xanthine, paraxanthine, uracil, thymine, allantoin, hydantoin, guanosine, adenosine, cytidine, and nucleic acids from the thymus gland and from yeast were examined. In many cases, there was noted survival for from 30 hours to two weeks, often with "very marked improvement." Unfortunately, however, in these early experiments only a very few birds (two or three usually) were used for each compound tested. Later developments of the testing of vitamin B have so conclusively demonstrated the pitfalls attending the curative pigeon test, such as spontaneous cures, heat cures, glucose cures, etc. (3), that these results have become discredited.

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[1] Presented at the Buffalo meeting of the American Chemical Society, September 2, 1931.

Testing human cases of beri-beri in the Philippines, Williams and Saleeby (4) found allantoin beneficial in certain cases, but thought it did not completely cure the disease. E. B. Vedder and R. R. Williams (5) in studying the products obtained in the fractionation of a concentrate from rice polishings, used both preventive and curative pigeon tests and tended to confirm the views of Funk (2). They found that a sample of Merck's nucleic acid did not protect pigeons in 0.5 g. daily doses, and attributed this to a destructive effect of the alkalies used in the preparation of the nucleic acid. They believed the curative base more likely a pyrimidine than a purine, and thought it might exist in food as a constituent of nucleic acid.

Schaumann (6) was also at one time of the opinion that some phosphorus compound, possibly related to nucleic acid, is responsible for antineuritic activity. Ganassini (7) has more recently reported the separation of a highly potent powder from the hydrolytic products of yeast nucleic acid.

In later and more elaborate attempts to isolate the vitamin, Funk (8) failed to obtain an active substance, isolating only adenine and nicotinic acid. J. C. Drummond (9) found by a test on rats that neither adenine nor nucleic acid (from yeast or spleen) possessed growth-promoting properties. Funk and Dubin (10), employing yeast growth as a test for vitamin B, failed to find any stimulation of growth by the addition of a mixture of ten purine and pyrimidine bases.

Williams (11), in later work, tested a number of hydroxypyridine derivatives, and from the variations in the results of his curative tests was led to the view that tautomerism was responsible in some way for the vitamin action. With A. Seidell (12), he devoted considerable attention in 1916 to the crude adenine fraction of autolyzed yeast. This proved actively antineuritic, but its activity was rapidly lost on purification. They advanced the view that some isomer (or tautomer) of adenine is responsible for the physiological activity. They further stated that the inactive, recrystallized adenine could be made to acquire antineuritic properties by fusing it *in vacuo*, or by heating it with sodium ethylate at 180° C. Here again curative pigeon tests were used, so that the experiments (in the results of which there were great variations) are open to the objections which have been raised to Funk's early work.

Williams and Seidell added as contributory evidence for the induction of a change in adenine by their treatments, the production of a deep-blue color by the use of the Folin phosphotungstate test for phenols (13). This test was given only by the treated adenine, and by the active impure adenine first obtained from the autolyzed yeast. After the activity had been lost on recrystallization, the adenine no longer responded to the color test. They believed that this color test, which was given intensely by autolyzed yeast, paralleled in general the physiological activity of the fractions obtained from it.

Carl Voegtlin and G. F. White (14) agreed that, while this color reaction is by no means specific for vitamin B, its intensity does in general parallel the activity of antineuritic concentrates. They found, however, that adenine when treated as described by Williams and Seidell (12) gave only moderately positive or negative tests with the reagent, and failed to acquire any antineuritic power when administered to polyneuritic pigeons. Similar results were arrived at by A. Harden and S. S. Zilva

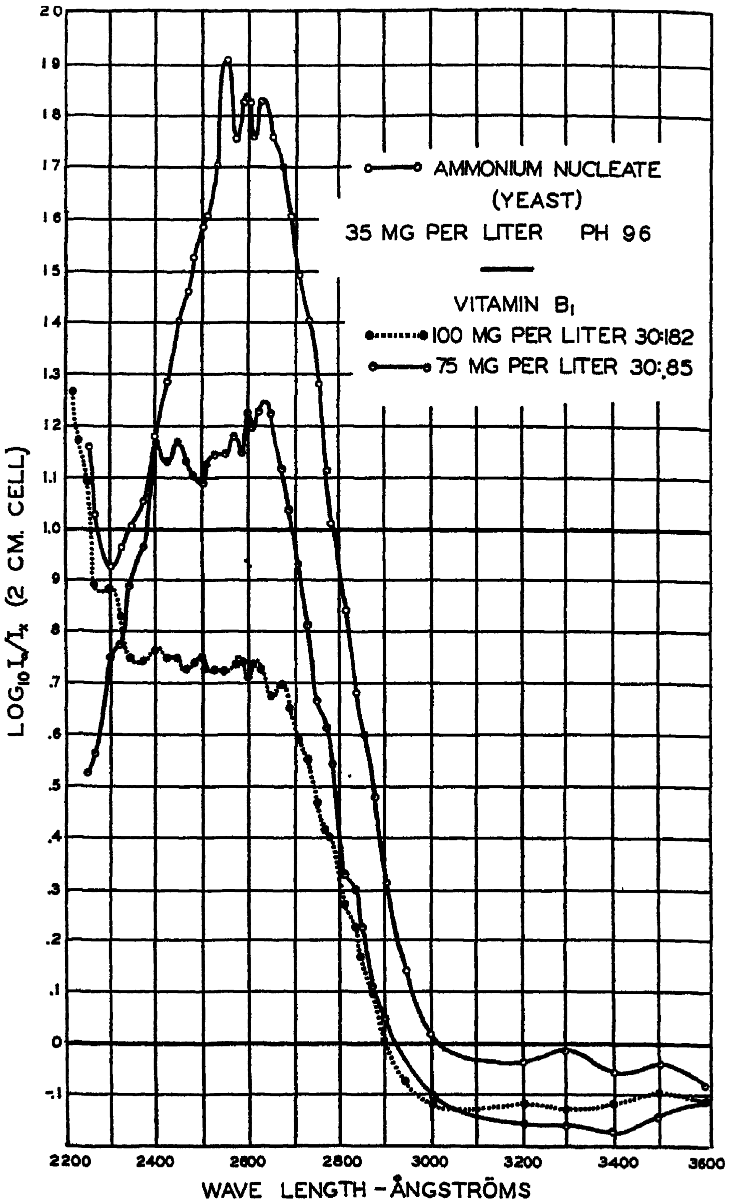

Fig. 1—Ultra-Violet Absorption of Two Vitamin B Concentrates, Supplied by Dr. Atherton Seidell, and of Yeast Nucleic Acid.

(15), who found that five pigeons treated with adenine which had been heated with sodium ethylate died. Three, however, showed a slight transient improvement before death, and one remained alive for six days. Williams (16) has also claimed to have obtained some degree of protection with trimethyluracil and with 4-phenylisocytosine.

The occurrence of nucleic acid derivatives in concentrates of high potency has been indicated by other more recent workers who have attempted to isolate the antineuritic vitamin by various procedures. Osborne and Wakemen (17) in 1919 studied the distribution of nitrogen in their highly active fraction II, and found relatively large proportions of purine nitrogen, indicative of nucleic acid. Jansen and Donath (18) in 1926 obtained from rice polishings a highly-potent, crystalline product, which they believed to contain an imidazole or pyrimidine ring, since it gave a positive Pauly's diazo reaction. Eijkman (19) also found that the Jansen-Donath preparation gave a positive blue color test with the Folin-Macallum phosphotungstate reagent.

Damianovich (20) in 1922 reported the only spectrographic study of vitamin B-containing products which we have been able to locate. No quantitative measurements were made, the absorption limits only being given as observed in photographs through various thicknesses of autolyzed yeast, solutions of fullers' earth adsorbates from autolyzed yeast, or various Osborne-Wakeman yeast concentrates. In view of the fact that these materials had not been subjected to any elaborate scheme of purification to remove the nucleic acid, it is perhaps not surprising that Damianovich concluded his observations to indicate the presence of a purine or pyrimidine ring.

THE ULTRA-VIOLET ABSORPTION SPECTRA OF VITAMIN B CONCENTRATES

Since frequent attempts have been made in the past to correlate the activity of vitamin B with various nitrogenous components of nucleic acid or with isomers derived from them, it seemed desirable to seek spectrographic evidence for the presence or absence of such substances (or related products) in the most highly-purified vitamin B preparations available.

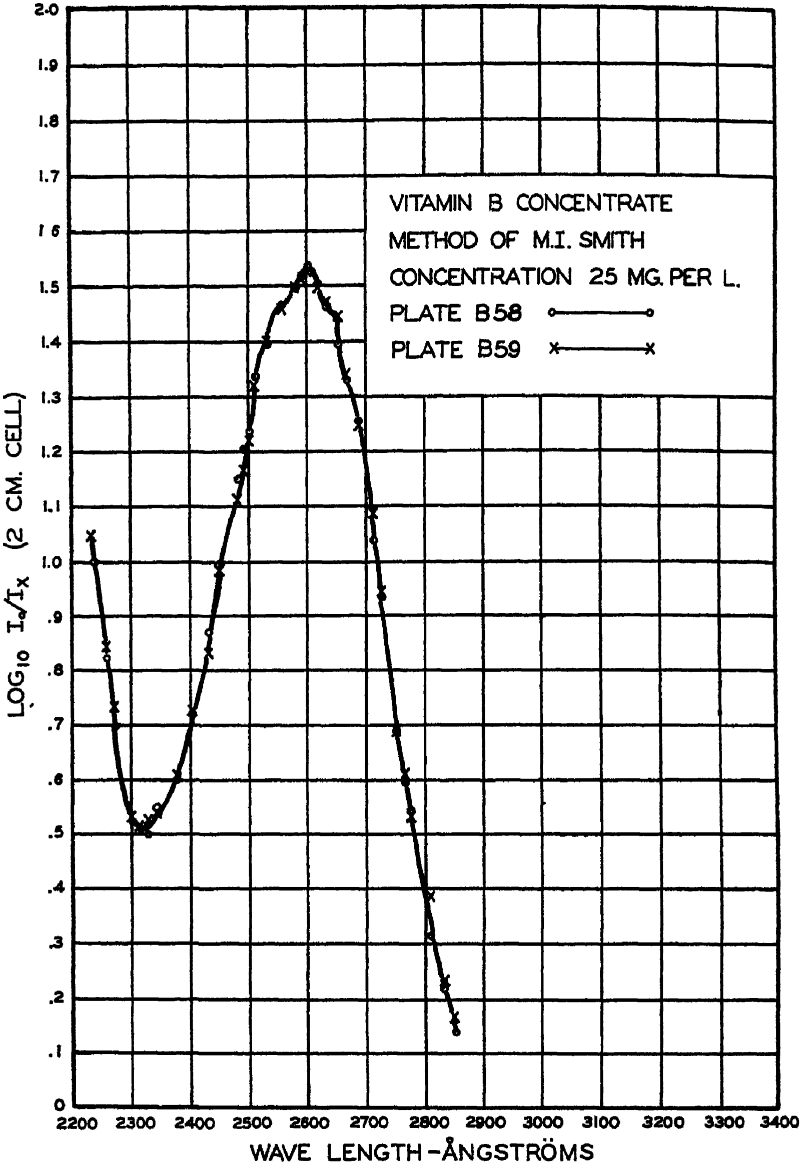

Fig. 2—Ultra-Violet Absorption of a Vitamin B Concentrate Prepared in this Laboratory by the Method of M. I. Smith. The circles and the crosses represent two separate series of determinations, illustrating the accuracy of the method.

Through the kindness of Dr. Atherton Seidell we have been enabled to study the absorption of two of his preparations. The methods of purification he employed have been described

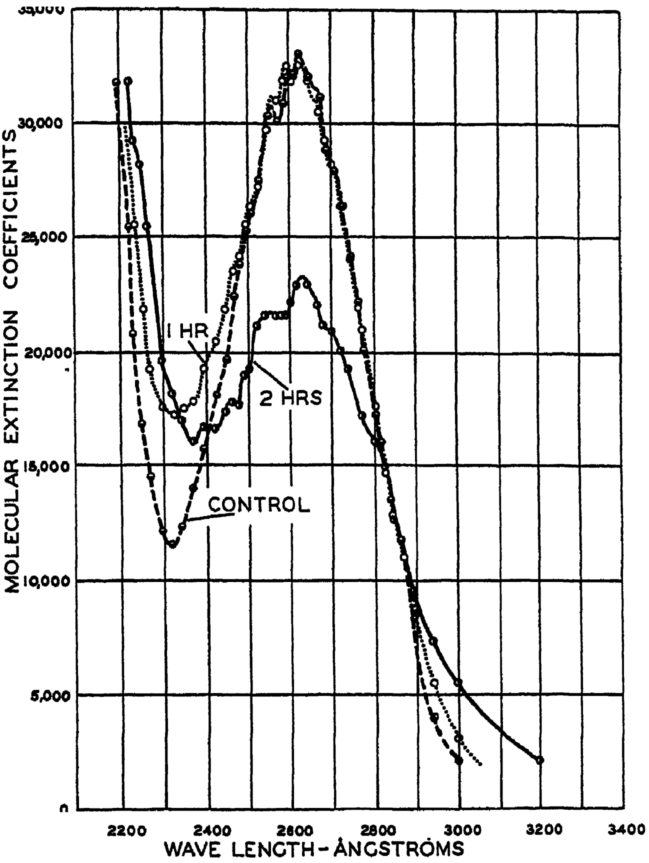

Fig. 3—Effect of Irradiating Thymus Nucleic Acid with Full Ultra-Violet

by him in a series of papers (21). The samples gave the blue color test with the arsenophosphotungstate reagent very intensely. Dr. Seidell stated that preparation 30:85 cured polyneuritic rats in a dose of 0.05 mg., as determined by the assay method of M. I. Smith (22). The potency of the other preparation had not been determined.

Preparations obtained by Dr. Seidell usually contain from 7 to 11 per cent of nitrogen. The highest nitrogen content he obtained was 13.1 per cent, but this was not his most potent product, as it required 0.08 mg. of it to cure polyneuritic rats. His most active preparation, with a minimum curative dose of 0.03 mg., contained only 7 per cent nitrogen, indicating that the nitrogen content is not a criterion of potency.

Figure 1 shows the absorption of the two Seidell preparations, along with the absorption of the ammonium salt of a commercial sample of yeast nucleic acid, the latter having been chosen as fairly typical of the type of absorption we have obtained for purine-containing or pyrimidine-containing materials. There are evident features of similarity and of dissimilarity between the curves. The nucleate curve envelops the curves of the two B preparations, peaks at 2560, 2600, and 2630 Å appear in each, and each is fluorescent at wave-lengths longer than 3000 Å. On the other hand, while the nucleate curve shows a decreasing absorption from 2550 Å. to a minimum at 2300 Å., the curves of the vitamin preparations show additional peaks between 2300 and 2550 Å. with extinction values of about the same magnitude as those from 2550 to 2650 Å.

Figure 2 presents an absorption curve of a vitamin B concentrate prepared in this laboratory by the method of M. I. Smith (22). Its curative dose has not been determined; Smith found each of two similar preparations to have a minimum curative dose of 1 mg. This preparation has an absorption more similar to that of yeast nucleic acid than to that of the Seidell products.

In discussing the absorption of a partially purified preparation, one must consider that it may be due to: (a) the substance in question, (b) impurities, or (c) both. These curves are presented with a full realization of that fact. Until a pure vitamin B preparation is studied, it is not possible to be certain that one has measured the absorption of the active material. It is, however, possible that parallel biologic and spectrographic tests on samples of different potencies might yield evidence as to which part of the absorption is due to impurities and which to the active material.

The similarities of the nucleate and concentrate curves in the 2600 Å. region lead us to conclude that there is at least strong evidence for the presence of purine or pyrimidine substances in the concentrates.

The dissimilarities in the absorption in the 2300 region may be due to the presence of absorbing substances other than purines or pyrimidines, or they may be due to a constitutional modification of the purines or pyrimidines. In regard to the latter possibility we may refer to our previous work (1), which indicated that irradiating uracil with ultra-violet light increases its absorption about the minimum at 2300 Å. That paper also showed that the absorption of thymus nucleic acid was markedly increased between 2300 and 2550 Å. by irradiation (Figure 3).

Prolonged irradiation with the unfiltered radiations from a quartz mercury arc leads first to this increased absorption and later to a decrease, which results eventually in the practically complete disappearance of selective absorption. To obviate this, and because of other considerations to be discussed elsewhere (such as the results of our colleagues on the growth-promoting effects of ultra-violet radiations (23)), we have employed a filter which eliminates most of the radiations of wavelength shorter than about 2960 Å.

Irradiation of an aqueous solution of thymus nucleic acid (35 mg. per liter) under this filter in a filled cell or in quartz test tubes under nitrogen caused a progressive increase in selective absorption (Figure 4), while the destruction of the compound showing selective absorption was largely eliminated. The increase in absorption due to the longer-wave radiations is particularly manifest in the region between 2300 and 2500 Å., and the nature of the change is such as to increase the resemblance between the absorption of the nucleic acid and that of the vitamin B preparations. This result suggests that the latter may contain a material related to the pyrimidine ring, and which has an absorption similar to that of the product produced

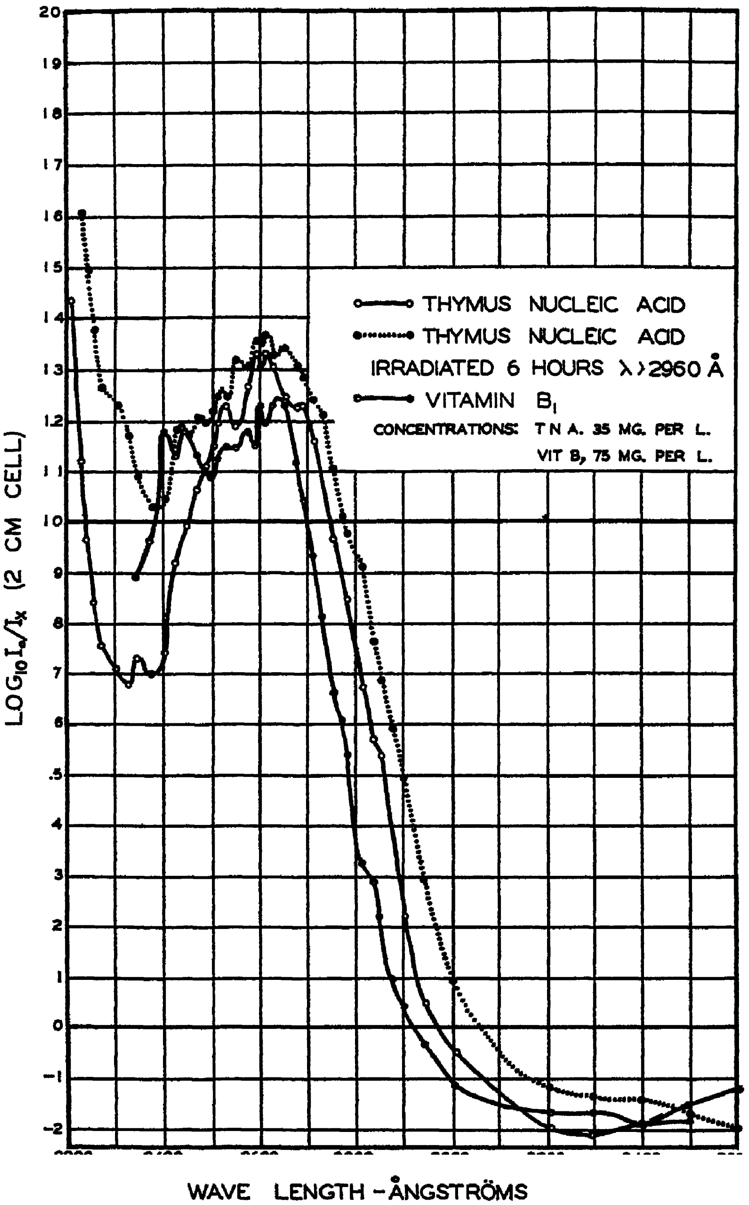

Fig. 4—Effect of Long-wave Ultra-Violet Irradiation on the Absorption Spectrum of Thymus Nucleic Acid.

by the irradiation of nucleic acid by radiations longer than 2960 Å.[1]

Additional evidence that irradiation induces constitutional changes in pyrimidines is afforded by our observation[2] that uracil and adenine when irradiated acquire the ability to reduce arsenophosphotungstic acid with the formation of a blue color. This test, it will be recalled, has been held by some authors to be an index of the vitamin B content of concentrates; it is given intensely by the highly-potent Seidell and Jansen-Donath preparations.

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[1] Experiments on the biological effects of irradiated nucleic acids are in progress.

[2] Unpublished.

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A COMPARISON OF THE COLORIMETRIC, SPECTROGRAPHIC, AND BIOLOGICAL METHODS FOR THE DETERMINATION OF VITAMIN A.[1]

By ROBERT J. NORRIS

SUMMARY

Seven cod-liver oils were assayed biologically, colorimetrically at 572 and 606μμ, and spectrographically at 325μμ. The physical tests showed fairly good correlation among themselves, but deviated from the biological tests in some instances by a considerably greater amount than the probable biological error. The 572 and 606μμ color test values agreed equally well with the absorption at 325μμ. It is concluded that biologic tests are the only reliable method for the vitamin A assay of cod-liver oils at the present time.

In a previous report from this laboratory (1) attention was called to the wide discrepancy shown between the biological and colorimetric values in following the inactivation of vitamin A by ultra-violet radiations. This study led us to the conclusion that the colorimetric method does not accurately represent the vitamin A content of liver oils under all conditions. The majority of workers, however, have inclined to the belief that the color test, while at times yielding inconsistent results, is fairly reliable as a measure of vitamin A content, and many of the earlier investigators believed these inconsistencies were the result of inherent errors in the biological assay.

REVIEW OF THE LITERATURE

Steudel and Peiser (2), Jones *et al* (3), Bailey, Cannon, and Fisher (4) have failed to find complete agreement between colorimetric and biological methods of assay. Hawk (5) reported in his criticisms of this test that exposure of cod-liver oil to air and radiations actually increased the blue color yield with the antimony trichloride reagent. Drummond (6) attempted to confirm Hawk's observations, but was unable to obtain similar results. Norris and Church (7) were able to confirm Hawk's earlier work, and the recent work of Heilbron, Gillam, and Morton (8) has added further confirmation to Hawk's findings and demonstrated that the increased blue color obtained with ageing is due to a marked increase of the chromogen producing a band at 606$\mu\mu$.

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[1] Presented at the Buffalo meeting of the American Chemical Society, September 1, 1931.

Brode and Magill (9), in a critical study of this color test, found that it was possible to produce a band at 608$\mu\mu$ or 578$\mu\mu$ or both by changes in concentration or temperature, or by the addition of certain substances to the oils. They are in agreement with Heilbron, Gillam, and Morton (8) in regard to the presence of more than one chromogen in liver oils. They suggest, however, as an alternative explanation, that changes in temperature, concentration, etc., may produce changes in the molecular configuration of the chromogenic substance.

From these observations it is apparent that there is no definite unity of opinion regarding the color test. The effects of concentration and temperature of the reagent, the changes brought about by added substances, and the question as to which band actually may represent vitamin A, cast uncertainty on the reliability of this reaction as a quantitative procedure.

Morton and Heilbron (10) called attention to the 328$\mu\mu$ absorption band in the ultra-violet spectrum, which seems to be constantly associated with the presence of vitamin A. Many other workers have confirmed these findings, indicating that this band may be a quantitative as well as qualitative measure of the vitamin A content of liver oils.

Heilbron, Gillam, and Morton (8), in a critical study, compared the ultra-violet band at 328$\mu\mu$ with the color bands in the visible spectrum at 572$\mu\mu$ and 606$\mu\mu$. They found evidence of better correlation between the 572$\mu\mu$ and 328$\mu\mu$ bands than between the 606$\mu\mu$ and 328$\mu\mu$ bands. Substantially the same findings were reported by Lovern, Creed, and Morton (11), using monk-fish liver oils.

Coward, Dyer, Morton, and Gaddum (12), in a study of the correlation between biological, colorimetric, and spectrographic methods of vitamin A assay, came to the conclusion that the discrepancies between the physical and biological methods are much greater than the sampling error of the biological test. They found the absorption values at 328$\mu\mu$ to give the closest correlation with biologic values, and fresh oils showed the best agreement among the three tests. Their work established the fact that with carefully controlled biologic technic the maximum error to be expected is approximately 30 per cent. Their published results show a remarkable correlation between the colorimetric and ultra-violet absorption spectrum methods. The correlation, however, between their physical and biologic results is so poor that it is quite obvious, judging from their work, that it is at present not possible accurately to determine vitamin A except by the biologic assay. It would appear that neither the colorimetric values nor absorption at 328$\mu\mu$ accurately measure the vitamin A content of liver oils. Their findings are in agreement with our experimental results reported in this and previous papers.

EXPERIMENTAL

Six standard brands of cod-liver oil were assayed biologically, colorimetrically, and spectrographically at 325$\mu\mu$.[1]

The colorimetric measurements were made at 606$\mu\mu$ and 572$\mu\mu$. The biologic and colorimetric techniques were essentially the same as those reported in our previous work (1).

The ultra-violet absorption spectra of the oils were measured with a quartz spectrograph, using an improved match-point technique, the maximum deviation of which (as determined by repeated observations on the same samples) is about 2 to 3 per cent of the extinction coefficient values. This technique will be described in detail in a paper to appear later.

One hundred fifty rats were employed in the biologic assay (twenty-five for each oil, which was fed at five dosage levels, and five for each dose). The feeding experiments were continued

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[1] The absorption at 325$\mu\mu$ was used in this paper instead of that at 328$\mu\mu$ because the type of spectrographic source used made it possible to determine the 325$\mu\mu$ absorption with somewhat greater accuracy Our results in many measurements show that the 325$\mu\mu$ and 328$\mu\mu$ absorption are closely parallel.

The ultra-violet absorption data were kindly supplied by my colleague, J. R. Loofbourow.

TABLE

Biologic, Colorimetric, and Absorption Spectra Assay of Cod Liver Oil

Oil Number	Number of Rats in Group	Precent Surviv-ing	Daily Dosage, mgs.	Average Weekly Gain, gms.	Daily Minimum Dose, mgs.	Vitamin A Assay			
						Biologic	Color Test $\frac{1}{CD}$ $\text{Log}_{10}(I_o/I_x)$, 1%, 1cm		Absorption at 325$\mu\mu$ $\frac{1}{CD}$ $\text{Log}_{10}(I_o/I_x)$, 1%, 1cm
							572$\mu\mu$	606$\mu\mu$	
7	25	0 0 60 80 60	0.5 1.0 2.0 4.0 8.0	0 0 0 9 13	4	1.0+	0.81	1.00	0.860
4	25	0 40 0 60 60	0.5 1.0 2.0 4.0 8.0	0 0 0 6.5 6.2	4	1.0	0.905	1.12	1.275
2	25	0 0 20 60 80	0.5 1.0 2.0 4.0 8.0	0 0 0 0 12	8	0.5+	0.59	0.68	0.565

TABLE I—Continued

Oil Number	Number of Rats in Group	Precent Surviving	Dailey Dosage, mgs.	Average Weekly Gain, gms.	Daily Minimum Dose, mgs.	Vitamin A Assay			
						Biologic	Color Test $\frac{1}{CD}\mathrm{Log}_{10}(I_o/I_x)$,1%,1cm		Absorption at 325$\mu\mu$ $\frac{1}{CD}\mathrm{Log}_{10}(I_o/I_x)$, 1%,1cm
							572$\mu\mu$	606$\mu\mu$	
3	25	0 20 20 40 80	0.5 1.0 2.0 4.0 8.0	0 0 0 0 10	8	0.5	0.915	1.03	1.06
5	25	0 0 20 0 40	0.5 1.0 2.0 4.0 8 0	0 0 0 0 9	8	0.5—	Not determined	0.44	0.475
1	25	0 40 0 40 40	0.5 1.0 2.0 4.0 8.0	0 0 0 0 0	More than 8	Less than 0.5	0.397	0.51	0.455
6	No Biological Data					.....	0.46	0.64	0.485

for five weeks after vitamin depletion had occurred, and the average weekly group gain was determined for each dosage level. The minimum dose was taken as the smallest quantity producing a definite growth increment over this period.

Table I shows the results of the biologic assay, the color test extinction measurements at 606$\mu\mu$ and 572$\mu\mu$, and the ultra-violet extinction measurements at 325$\mu\mu$.

In Table II, the relative potencies of the oils are expressed as percentages, taking oil 7 (the most potent one biologically) as 100. This illustrates the marked discrepancies between the biological and physical methods of assay. Oils 3 and 5, for example, show practically the same potency biologically, while the color test and absorption at 325$\mu\mu$ indicate a potency twice as great for oil 3. Oil 2 has slightly higher potency than oil 3 according to the biological assay, but the physical methods indicate it to be only about half as potent. In the case of oils 1 and 5, the ultra-violet absorption data agree with the relative order of biological potency, while the 606$\mu\mu$ color test band indicates that oil 1 has the greater potency.

TABLE II

Relative Potencies of Various Oils (Oil No. 7 taken as 100)

Oil No.	Biological Assay	Colorimetric Assay		U. V. Absorption 325$\mu\mu$
		606$\mu\mu$	572$\mu\mu$	
7	100	100	100	100
4	100−	115	111	149
2	50+	69	73	66
3	50	103	115	123
5	50−	47	...	55
1	30	55	52	53
6	.....	64	56	57

DISCUSSION

The results reported here confirm our previous findings (1) and those of other workers (12), that there is a marked discrepancy between the biological and physical vitamin A assays of various cod-liver oils.

The colorimetric and ultra-violet absorption assays show a considerable degree of correlation, suggesting a relationship between the chromogen or chromogens responsible for the antimony trichloride reaction and the absorption at 325$\mu\mu$. This agreement, however, may be entirely fortuitous. In following the progress of the inactivation of vitamin A in oils under ultra-violet radiations (13), we noted that the rate of destruction of the substance or substances responsible for the 325$\mu\mu$ band did not agree quantitatively with either the colorimetric or biological values run as parallel tests.

Contrary to the results of Heilbron, Gillam, and Morton (8), we have found no essential difference in the correlation of the 572$\mu\mu$ and the 606$\mu\mu$ color tests with the ultra-violet absorption. Referring to Table II, one finds that in the case of oils 7, 2, and 6, the 572$\mu\mu$ extinction agrees better with the ultra-violet absorption, while in the case of oils 4, 3, and 1, the 606$\mu\mu$ color values agree better (oil 5 cannot be considered since its 572$\mu\mu$ color test value was not measured). Only a limited number of oils has been studied, however, and it is possible that if many oils were measured, the majority would show better correlation between the 572$\mu\mu$ color test and the ultra-violet absorption.

It is quite logical to assume, as has been suggested by other workers, that there may be a number of substances other than vitamin A present in cod-liver oils which are responsible for a part of the colorimetric and ultra-violet absorption. In support of this viewpoint, one finds that the ultra-violet absorption of oil 5 is quite different from that of the other oils. Further, as stated above, during inactivation by ultra-violet the rate of destruction of the ultra-violet absorbing materials and the chromogens responsible for the color test show significant differences, indicating (at least for certain oils) that the correlation between the colorimetric values and the absorption at 325$\mu\mu$

is not consistent. It might be proposed that all three assay methods may be, in some oils, measuring essentially different fractions of a mixture of closely related compounds.

CONCLUSIONS

1. It has been shown that there is no consistent correlation between physical and biological methods of assay for vitamin A in a group of cod-liver oils.
2. Our previous work (1) and the results of other investigators (12) have shown that the error between the biological and physical measurements is much greater than the probable error in the biological tests.
3. The colorimetric and spectrographic methods of assay for vitamin A are in fairly close agreement for the oils studied.
4. The correlation between the spectrographic and colorimetric tests appears equally satisfactory with these oils whether measurements are made at 572$\mu\mu$ or 606$\mu\mu$.
5. Occasional oils have been found in which there is a decided difference between the absorption values and the colorimetric values.
6. In our estimation, the biologic methods of assay for vitamin A remains the only reliable method at the present time.

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VITAMIN D—A REVIEW

PART I—ULTRA-VIOLET ABSORPTION SPECTRA IN RELATION TO VITAMIN D (CONTINUED).

BY JOHN R. LOOFBOUROW

SECTION C—ULTRA-VIOLET ABSORPTION OF OILS (CONCLUDED).

SUMMARY

Olcott and McCann (83) suggested that the 2800 Å. maximum found in various oils, in ergosterol, and in carotene may be due to a similar chemical configuration in these substances. Ahmad (84) published spectra of the caecum fat from rats, showing evidence of the production of 3280 Å. absorption from carotene in the intestines of the animals. Lovern and Morton (85) published the spectrum of monk-fish liver oil. Heilbron, Gillam, and Morton (86), Coward, Dyer, Morton, and Gaddum (87), Lovern, Creed, and Morton (88), Gillam and Morton (89), and Norris (90) investigated the 3280 Å. maximum in oils in an attempt to determine its quantitative relationship to color and biologic tests for vitamin A.

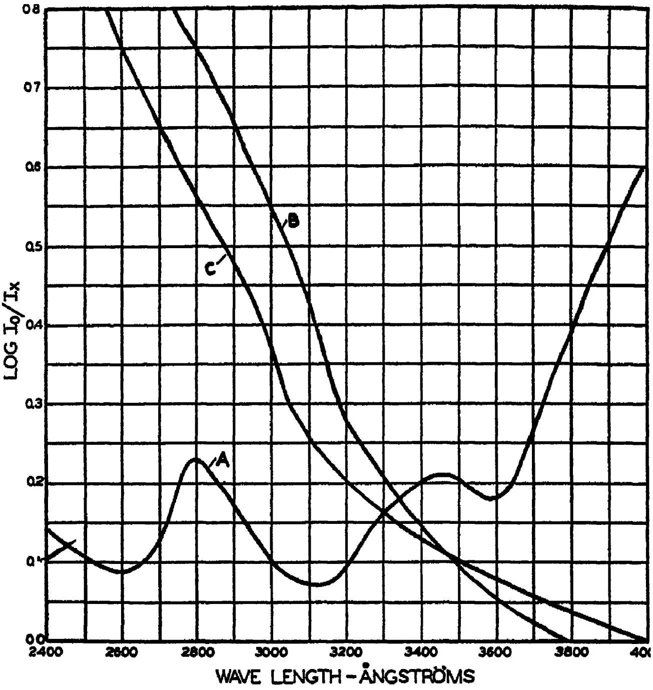
Since the publication of the last part of this review, dealing with the ultra-violet absorption of oils, several papers regarding the absorption of oils and their constituents have appeared which should be mentioned briefly before passing on.

Many workers whose papers have already been reviewed found a band in oils at about 2800 Å. This band has been attributed by some to ergosterol or vitamin D. Others have noted it, but have not attempted to designate its origin. Olcott and McCann (83; 1931) make the following interesting statement: "Both carotene..... and its isomer, lycopin (unpublished observations), have a band at 280 mμ, although lycopin differs from carotene in lacking the band at 345 mμ. One of the three important bands of ergosterol is at 280 mμ. Although it may be a chance occurrence, it is possible that some molecular configuration, common to carotene, lycopin, ergosterol, and the unknown substances in fish and animal oils, is responsible for the absorption at 280 mμ."

Whether or not this is true, it seems probable that part or all of the absorption of oils attributed by various workers to ergosterol or vitamin D may be due to carotene.

Olcott and McCann's curves for carotene, achroocarotene, and oxidized carotene (Ab6) are reproduced in Figure 38.

These authors used ultra-violet absorption spectra to determine if vitamin A could be formed from carotene *in vitro* by incubating it with ground up rat livers or other liver extracts. Their absorption curves, reproduced in Figures 39 and 40, do show the formation of a band near 3280 Å. by such treatment



Chloroform solutions; curve A, 1:75,000; curve B, 1:10,000, curve C, 1:50000; cell thickness not stated (GG).[1]

Fig. 38—Absorption of Carotene (curve A), Oxidized Carotene (curve B), and Achroocarotene (curve C). [Olcott and McCann (83; 1931)].

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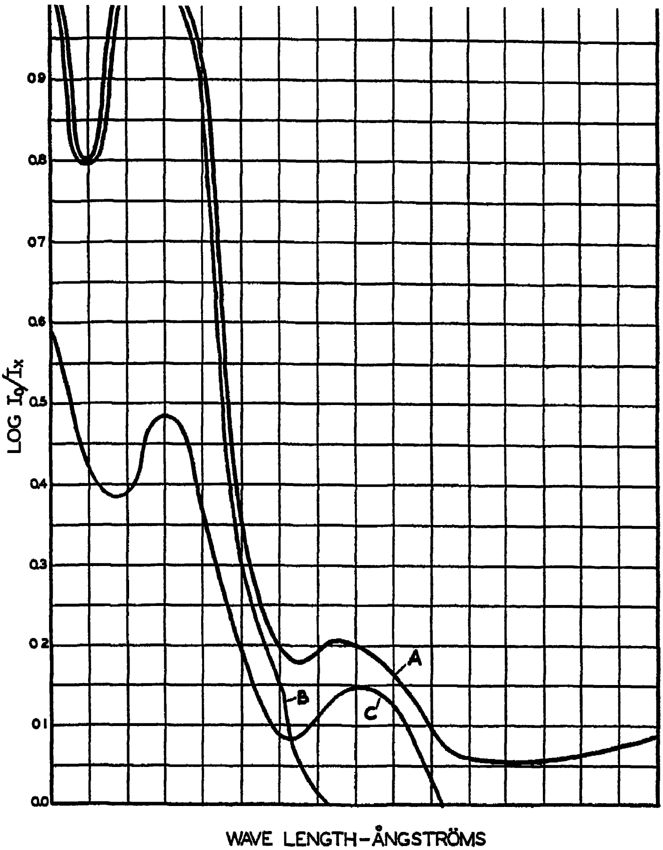
[1] These key letters refer to the methods of plotting curves used by authors. A table of the key letters will be found in the first section of this review [*Bull. Bas. Sci. Res.* **3** (1931): 105].

of carotene. They attribute this transformation to an enzyme, carotenase, which their experiments indicate to be destroyed by heat.

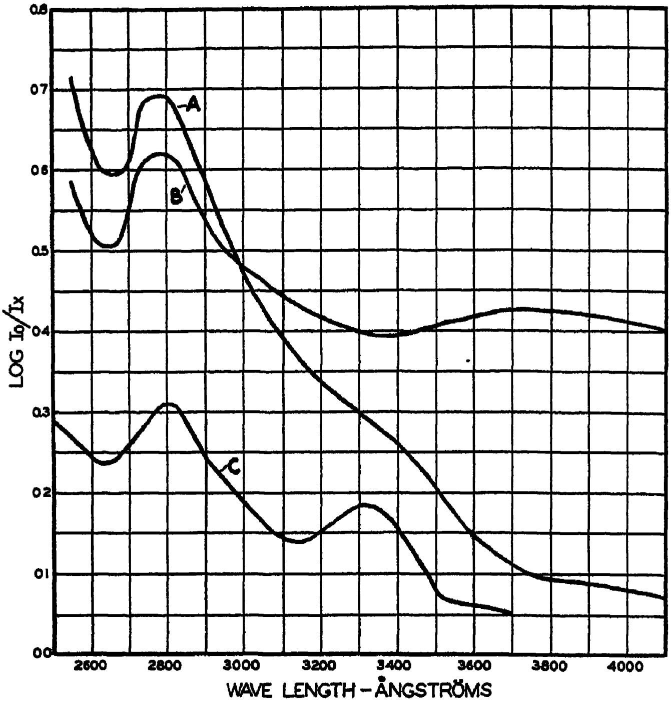


Chloroform solutions; curves A and B, 1:300; curve C, 1:500; cell thickness not stated (GG). See footnote page 258.

Fig. 39—Absorption of Unsaponifiable Liver Lipids of Various Rats [Olcott and McCann (83; 1931).]

Curve A, liver of rat 347 incubated in vitro with carotene; Curve B, liver of rat 331, incubated without addition of carotene; Curve C, liver of rat 333, carotene added to diet.

Ahmad (84; 1931) examined spectrographically the caecum fat from rats kept on an A-free diet and fed carotene. He found distinct carotene peaks, and also some evidence of a 3280 Å. band, which he believed might indicate the synthesis of vitamin A from carotene in the intestines of the animals. A curve of the caecum fat is reproduced in his paper. He obtained some



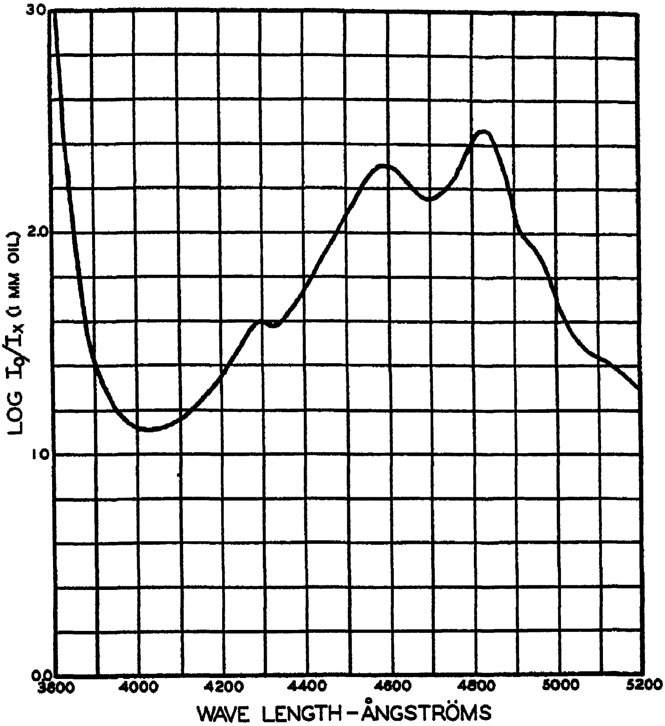
Chloroform solutions, concentrations not known, cell thickness not stated (GG). See footnote page 258.

Fig. 40—Absorption of Ether-Soluble Constituents of Liver Extracts. [Olcott and McCann (83; 1931)].

Curve A, liver extract; curve B, heated liver extract after incubation with carotene; curve C, unheated liver extract after incubation with carotene.

indication of the production of a 3280 Å. band in carotene cultured with intestinal bacteria, but could find no evidence of the formation of this peak in carotene incubated with rat livers *in vitro,* in contradistinction to Olcott and McCann's results.

Lovern and Morton (85; 1931) studied the absorption of monk-fish liver oils because of a peculiar pink pigmentation which they found in monk-fish livers. Their one curve is reproduced in Figure 41. It does not extend into the ultra-violet far enough to include ergosterol peaks, and they do not state whether such peaks were found. In the visible region it shows maxima which they believe to suggest the presence of a carotenoid pigment, although they point out that the positions of the maxima are not exactly the same as those of carotene dissolved in oil.



Absorption of oil measured directly in 0.5 mm. layer (GGI). See footnote page 258.

Fig. 41—Absorption of Monk-Fish Liver Oil [Lovern and Morton (85; 1931)].

Heilbron, Gillam, and Morton (86; 1931) mention the

2800 Å. maximum in their recent discussion of liver oils and concentrates, but do not discuss the nature of the substance responsible for it.

These authors and Coward, Dyer, Morton, and Gaddum (87; 1931), Lovern, Creed and Morton (88; 1931), Gillam and Morton (89; 1931), and Norris (90; 1931) have studied the absorption of many liver oils at or near 3280 Å. in order to determine more accurately the relationship of this peak to the color and biological tests for vitamin A. This work is reviewed in some detail in Norris' paper (90; 1931).

Practically all workers still seem to hold to the viewpoint that the 3280 Å. band is characteristic of vitamin A and has no relation to vitamin D. Some claim that the 572 mμ color test peak for vitamin A is correlated more closely with the 3280 Å. ultra-violet band than the 606 mμ color test peak. The evidence does not seem very conclusive, however, as far as liver oils are concerned.

The next section of the review will deal with the changes brought about in the absorption of ergosterol by irradiation with ultra-violet, with the ultraviolet absorption of compounds chemically related to ergosterol, and with the probable absorption of vitamin D.

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THE EVALUATION OF VITAMIN B_1 CONCENTRATES BY THE RAT CURE METHOD

By FRANCIS F. HEYROTH

SUMMARY

The literature regarding the assay of vitamin B_1 is reviewed in detail. Curative rat tests as employed on several B_1 concentrates are described, and the requirements for accurate results in assays by this method are outlined.

It has recently been shown in this laboratory (1) that the ultra-violet absorption spectra of certain admittedly impure vitamin B (chiefly B_1) concentrates appeared to indicate the presence therein of substances containing purine or pyrimidine rings. It was suggested that parallel biologic and spectrographic examination of samples of different activities might yield information as to whether the absorption might be ascribed to impurities or to the biologically active substances. Upon undertaking to determine the biological activity of the samples in question, it was found that a considerable number of assay methods was available. We present here a critical discussion of these methods, together with the data obtained regarding the activity of the concentrates studied.

GENERAL CONSIDERATIONS

The maintenance of weight, the promotion of growth, and the cure of polyneuritic symptoms have each been employed as measures of the various components of the vitamin B complex. The frequent occurrence of refection in rat growth experiments, and the inadequacies of the diets employed in most pigeon weight methods, together with the current uncertainties as to the contributions of the individual factors of the B complex alone, or in various combinations, to growth, led us to adopt a curative test. R. A. Peters (2) stated recently, "As an absolute control, a curative test is better than one employing maintenance or growth, though the latter can be used under conditions where all other necessary factors are present in excess."

The pigeon has been most used in curative tests. Erroneous results, frequently encountered by previous workers, may be obviated only by the adoption of some such elaborate precautions as those prescribed by Kinnersley, Peters, and Reader (3) after experience with several thousand birds. We employed the rat in our experiments since recently described rat cure assay methods appeared to offer much in convenience and accuracy. As there is much confusion in the literature as to the symptoms of vitamin B deficiency in the rat, an historical review of the observations dealing with it is presented.

POLYNEURITIC MANIFESTATIONS IN RATS

The first to describe rat polyneuritis seems to have been Schaumann (4), who in 1910 found that on a diet of horse meat "denatured" by heating to 120° C. with a 10% sodium hydroxide solution, rats exhibited paresis of one or both of the hind legs, and some animals showed circus movements. In the light of our present knowledge of the vitamin B complex, Schaumann's diet would seem to have been deficient in B_1, although not necessarily so in B_2. Among others to describe symptoms attributed to a deficiency of vitamin B in the rat were Langstein and Edelstein (5), Funk and Macallum (6), and J. C. Drummond (7). These workers noted polyneuritic symptoms only occasionally or incidentally.

F. Hofmeister, 1922 (8), was the first to succeed in producing such symptoms in a large percentage of his rats, and to describe them thoroughly. He fed a diet of casein, rice starch, cod-liver oil, lard, and salts, but gave no yeast. While this diet apparently lacked all members of the B complex, this was probably not really the case, since no mention was made of any measures having been taken to free the casein from such nutritive factors. As our observations agree closely with those of Hofmeister, a brief summary of his accurate description of the symptoms given here will make a complete description of our own rats in the experimental section unnecessary.

Four stages of the disease were discerned. A prodromal phase (A) of loss of appetite, diminishing weight, depression of spirit, and general inactivity, gradually increasing during about four weeks, was followed by (B) a stage of ataxia. This was marked by weak and incoördinated muscular movements, and disturbances in gait, which caused the animals to move with difficulty, and often to appear fixed to the spot at which they were placed. Progressive motion in many instances was by sliding or crawling, or the rats proceeded tremulously and with convulsive starts. From their sides or backs, many were unable to regain an upright posture. The rats often fell, and hesitated to jump from a moderate height. When returned to their cages, they sought dark corners. About half of such rats passed directly into a state of somnolence and extreme weakness, which led to death. The others went into (C) a spastic phase of varied symptoms. Prominent among these symptoms was a tonic extension of the extremities, one or both of the hind legs being stiffly abducted, or both being extended backwards. From their backs, the rats put forth such excessive efforts to right themselves as to roll for a considerable distance. In some, the spasticity extended to the fore legs, the head was retracted, and the back arched. Cramps often appeared as the result of intentional movements, misdirected in intensity and duration; they could be induced by external stimuli. In a few rats, there were tremors, twitches, and swinging movements of the head, and, although rarely, epileptiform convulsions. After a few hours or days, condition (C) passed into a phase (D) of paralysis, weakness and coma, ending in death. The spastic stage occurred only in those animals which received some small addition of vitamin B-containing material to the diet; otherwise (B) passed directly into (D). The edema of the feet observed in three of thirty rats was ascribed to vasomotor disturbance.

Hofmeister showed by microscopic investigations that in the rat degeneration of the peripheral nerves could but rarely be found. Instead, multiple hemorrhages were noted in the brain, especially in the cerebellum and brain stem. These were accompanied by degeneration of the central nervous elements, yielding a picture characterized as "brain purpura", analogous to the hemorrhagic encephalitis encountered in chronic lead poisoning. The term "polyneuritis" is thus, at least in this animal, a misnomer, but it is in such wide use that one must retain it.

Chick and Roscoe (9) were much less successful in inducing the nervous symptoms in rats, their animals usually dying of inanition in from three to four weeks. This may have been because, unlike Hofmeister, they purified their casein by alcohol and ether extractions. Goldberger, Wheeler, Lillie, and Rogers (10) in 1926, after showing the need of a thermostable yeast factor in rat nutrition, found that even when this was supplied in the absence of the antineuritic factor, beriberi did not develop in all rats. Sandels (11) seems the first to have definitely sought to supply B_2 in studying rat beriberi by including in his basal diet 15% of dry bakers' yeast, autoclaved three hours at 125° C. Even on this diet, however, the rats declined rapidly and died in about 38 days, rarely becoming paralyzed. But when small amounts of the antineuritic factor, insufficient for protection, were added to the diet in the form of an 85% alcoholic extract of whole wheat, the typical nervous symptoms developed almost without exception. In some cases a subacute or chronic form of the disease persisted several weeks. It was thus his opinion, as it had been that of Hofmeister, that in the complete absence of the vitamin, the nutritional failure is so great that the animals die before the nervous symptoms become manifest. An analogous condition seems to exist in regard to the symptoms of the B_2 deficiency manifested in the rat [Chick and Copping (12)].

THE RAT CURATIVE TEST

That the cure of the nervous manifestations of the deficiency of vitamin B in the rat might be employed in the evaluation of the "antineuritin" content of yeast concentrates was demonstrated by Hofmeister in his second paper (13) on "Qualitative Undernutrition", a paper which has received unmerited neglect from more recent investigators. "Antineuritin," like the "torulin" of Peters, was a term devised to indicate a curative factor for the neurological symptoms, quite irrespective of any effect it might or might not exert upon growth. In the ataxic or spastic stages, cures could usually be effected either by adding the vitamin-containing material to the food, or, and better, by subcutaneous injection. Rolling movements or epileptiform cramps were quieted within a few hours of an injection; the animals soon began to eat, and often appeared entirely normal within 24 hours. In three days at the most, adequate doses removed nervous symptoms, leaving only some degree of emaciation and muscular weakness. These cures were accompanied by a prompt increase in weight, often as much as one-third of the weight being gained in two or three days. After the cure had persisted for 8 to 14 days, there again occurred a loss of appetite and weight, and after a time the ataxic or spastic symptoms recurred. These could again be successfully treated as before, although this could not be repeated indefinitely. Rats in the final paralytic state (D) proved unsuitable for curative tests, as either the central nervous lesions had progressed so far as to be irremediable, or the animals died before cures could be expected. Occasionally in even much less severely affected rats, subcutaneous injections induced coma and death in from ten to thirty minutes.

The effect of various factors on the weight also could be observed, and the danger of the induction of fatal shock decreased, by making the injection during the period of sharp loss of weight, but before the onset of definite nervous symptoms. In such cases, the weight rose sharply for a few days after the injection, remained approximately constant for a period dependent upon the amount of the vitamin administered, and then again dropped sharply, beriberi symptoms then appearing. As a measure of the "antineuritin" injected, Hofmeister suggested what he called "recovery time", i.e., the number of days required for the weight to return to the value it had had when the injection was made.

Because of the failure of the other workers cited to develop uniformly the symptoms of beriberi in rats by dietary measures, the method of Hofmeister seems to have found no employment by others, until, in 1930, M. I. Smith (14), by adding 10% of autoclaved yeast to the diet, found it possible to produce a "specific polyneuritis" in from six to ten weeks in most young rats. He attributed his success to the more thermostable growth factor thus supplied. While such a factor is undoubtedly essential, the results of Sandels (11) and of Hofmeister (13) (compare the analogous findings of Chick and Copping (12) in the study of B_2 deficiencies) indicated that Smith's success may have been due, at least in part, to the presence in his diet of a small amount of vitamin B_1. Sebrell and Elvove (15) also presented evidence that the neurological symptoms of rats are those of an insufficiency rather than of the complete absence of the antineuritic vitamin. The source of this factor in Smith's work could have been either incomplete purification of the casein, or its incomplete destruction during the autoclaving of the yeast, the conditions of which were not stated. Possibly the other factors of the B complex, as the heat-labile B_4 of Reader (16), or the Y factor of Chick (17) may be essential. Not until the components of the B complex have been isolated, and administered separately and in various combinations, will it be possible to determine the exact conditions required for the production of experimental beriberi in the rat. Until then, the assay conditions must remain empirical. Sebrell and Elvove (15) found that when the casein used had been leached with water and then baked 24 hours at 140-142°, and further purified by alcohol and ether extractions, only one of three rats developed symptoms before death. As similar results were obtained when larger numbers of rats were used, they preferred leaching the casein with daily changes of acidulated water to baking it and extracting with alcohol and ether.

Smith (14), who presented weight curves confirmatory of those of Hofmeister, found an adequate dose of an antineuritic concentrate, intravenously administered, to alleviate the typical paralytic symptoms noticeably in from three to five hours, and unmistakably in 18 to 24 hours. The cure lasted from three to many days depending upon the dose. As a measure of antineuritic potency, he employed the minimal dose which produced a cure lasting for three days. Neither Hofmeister nor Smith described the skin lesions which appear in rats fed on a diet deficient in vitamin B_2.

In reporting on the value of the Smith method, Sebrell and Elvove (15) showed subcutaneous injections to be as satisfactory as intravenous ones. They employed only animals in which a convulsive seizure with extension of the extremities could be elicited by turning the animal on its back. In a series of injections of rather inactive concentrates, it was noted that as the minimal dose was approached, some rats failed to recover on doses curative for others. They, therefore, suggested that at least three rats be employed for a given dose, or that there be a minimum of 60% recoveries if more than three were employed. Occasional failures of doses considerably greater than the minimal curative dose were discounted. Cures were noted after five or six previous attacks, and in one instance even after the eleventh.

Kinnersley, Peters, and Reader (18) have also reported their experience with the curative rat test. They, however, used adult rats of approximately maximum weight. During 21 to 24 days on the experimental diet, the rats decreased in weight to about 100-120 grams, after which convulsions occurred rather suddenly, accompanied by partial paralysis, especially of the hind legs, the feet being splayed out. While some animals lived for a few hours in this condition, death frequently occurred in about 30 minutes. These workers stated that their "torulin" preparations administered by mouth often caused a marked improvement within twenty minutes, and always within two or three hours. "Convulsions and paralysis of the hind limbs appear to be the polyneuritic symptoms and are cured by vitamin B_1 in its purest form."

These symptoms were frequently accompanied by others they believed due to a deficiency of B_4, for the existence of which Miss Reader (16) had previously advanced evidence. She (19) believes that the addition of the B_1 concentrates (Kinnersley and Peters preparations) cures the typical polyneuritic symptoms without restoring weight, and views a "weak condition with swollen red paws, spastic gait, loss of coördination" as due to a B_4 deficiency. By administering, in addition, a B_4 preparation, the weight increased at a rate of 20 gm. per week, and all symptoms disappeared within three weeks.

The Oxford workers are thus of the opinion that the Hofmeister-Smith test may be split into separate assays for B_1 and B_4. After 3 to 4 weeks on a diet free from both B_1 and B_4 "typical polyneuritic convulsions occur, accompanied by great weakness and ataxia. The paws are often oedematous and very red between the digits, and there is partial paralysis mainly manifested in the hind legs. Now the animal is given a dose of vitamin B_1 and the convulsions and paralysis disappear within a few hours. If daily doses of vitamin B_1 (2 to 3 curative doses daily) are now instituted, the animal can be maintained in this condition, i.e., with a general muscular weakness, spastic gait, swollen red paws, and a tendency to sit in a hunched position, for about ten days. The weight, which has been falling rapidly up to this point, now ceases to fall, and maintains a steady level. If food and water are put at the far end of the cage, the rat can be persuaded to move, but frequently walks with a rolling movement and generally shows a lack of coördination. Such an animal is now ready for the vitamin B_4 test. The increase in weight is the first obvious sign that the sample under test contains B_4, but within two days the animal becomes much more active. At the end of a week the gait is apparently normal. If the sample tested does not contain vitamin B_4, the symptoms get rapidly worse and death occurs in 10 to 14 days."

Anomalous rats were admitted to require a slightly larger dose of one of the several factors (B_1, B_2, B_4) rather than an excess of all three. Neither of these two papers states the number of rats tested. Further, it is not entirely evident from the rather limited descriptions of the symptoms just how the various features of the symptom complex are assigned to B_1 and B_4 for Miss Reader (19) states (p. 1827), that spastic gait is among the symptoms apparently due to a specific lack of vitamin B_4, and (p. 1828) that "partial paralysis mainly manifested in the hind legs" disappears within a few hours of the administration of B_1. Except for those workers addressing themselves to the problem of the isolation of B_4, attempts to assay B_1 and B_4 separately by such methods seem premature.

EXPERIMENTAL

A THE PREPARATION OF THE CONCENTRATES

In the preparation of our concentrates from yeast we were interested solely in obtaining the rather small quantities required for biological tests and absorption measurements, and followed recognized procedures. No attempt was made to determine the effectiveness of the various steps, nor were we concerned with the problem of securing the best possible yields.

H_1, our first concentrate, was made by the procedure of M. I. Smith (14). A commercial dried yeast intended for poultry feeding was extracted by N/25 acetic acid. The precipitates obtained on successively adding the partially-evaporated solution to 95% alcohol, and later to acetone, were rejected. The active material was adsorbed on acid-treated norite at pH 4, and eluted by N/20 hydrochloric acid in 50% ethyl alcohol. Smith found each of two such preparations, evaporated to small volumes, to cure polyneuritic rats in 1 mg. doses.

The next two concentrates (H_2 and H_3) were prepared from fresh brewers' yeast kindly furnished by the Bruckmann Brewery of Cincinnati, Ohio. The procedure by which Jansen and Donath in 1926 (20) obtained from rice polishings a very active crystalline product (with a rat day dose of 5γ) was utilized with slight modifications.

Approximately ten lbs. of a thick yeast suspension, diluted with an equal volume of water, was heated to 90°, and allowed to cool to 55°. From the 7 l. of solution obtained after removing the coagulated material by a combination of filtration and centrifugation, the vitamin was adsorbed by about 200 gm. of Lloyd's reagent at pH 4.5. The active substances were removed from the precipitate by treatment with a saturated solution of barium hydroxide, and the filtrate immediately brought to pH 3 by the addition of a 50% solution of sulphuric acid. The solution obtained after removal of the barium sulphate was evaporated to 175 cc. before a fan.

In the presence of an excess of silver nitrate, a modified Kossel-Kutscher fractionation was carried out by separately collecting the precipitates obtained on the gradual addition of a saturated solution of barium hydroxide between the pH ranges of 3–4.5, 4.5–6.8, 6.8–8.0. The second of these fractions had been found by the Dutch workers to contain most of the vitamin. We determined the pH values only very roughly by moistening strips of an absorbing paper with the liquid and adding a drop of an indicator (bromcresol green, bromcresol purple, methyl red, and phenol red were employed). The colors were matched against those obtained by adding the indicators to strips of paper moistened with phthalate and phosphate buffers of known pH. In the preparation of H_2, the entire precipitate was obtained from pH 3 to 8, decomposed by hydrochloric acid, and the fractionation carried out on the filtrate from the silver chloride. As the precipitate between pH 6.8 and 8.0 was relatively small in amount, it was not kept separate but worked up with the fraction obtained between pH 4.5 and 6.8.

On acidifying with hydrochloric acid, a rather pleasant characteristic odor was noted; this was detected in all stages in which an alkaline solution was acidified. After determining the acidity of the solution by titration, there was added the calculated amount of concentrated sulphuric acid necessary to make the solution approximately equal in total acidity to that of a 5% sulphuric acid solution. This was precipitated by a 10% solution of phosphotungstic acid in 5% sulphuric acid. The vitamin-containing precipitate was dissolved in a 50% acetone-water mixture with the gradual addition of acetone until all had dissolved. The complex nature of the precipitate was clearly indicated; a large portion dissolved immediately, leaving a brownish-yellow, sticky, gummy material. This material dissolved completely on the further addition of small quantities of acetone. The phosphotungstates were reprecipitated by pouring the solution into a large excess of 5% sulphuric acid, again dissolved in an acetone-water mixture, and decomposed by adding barium hydroxide until phenolphthalein paper turned deep red. After removing the precipitate, the filtrate was at once acidified with sulphuric acid, both to precipitate the barium and to limit the destruction of the vitamin by alkali. With the attainment of acidity the characteristic odor was again noted. The filtrate from the barium sulphate was yellow. Barium chloride was added to remove the excess of sulphate, and after again filtering, the filtrate was evaporated, first on a water-bath, and then in a vacuum desiccator.

When dry, the residue was seen to contain some barium chloride crystals stained brownish yellow by organic matter. The organic portion was taken up in absolute ethyl alcohol, leaving undissolved the barium chloride. The addition of an alcoholic solution of platinum chloride produced a thick brownish yellow precipitate which was collected on a filter and allowed to dry. This was suspended in water, decomposed by hydrogen sulphide, filtered and evaporated, first on a water-bath, and then *in vacuo* over lime. The final product consisted of 3.6 mg. of yellow scales (H_2).

A repetition of the preparation gave a much larger yield of a product which, however, proved to have lower potency. In this preparation (H_3) the silver fractionation was not repeated.

In their original method, Jansen and Donath (20) dissolved the products left after the evaporation of the decomposed platinum precipitates in alcohol and by the addition of small amounts of acetone obtained a milky cloudiness which, after a few days, formed on the walls of the vessels a partly viscous, partly crystalline deposit. This deposit was again dissolved in alcohol and precipitated by acetone additions. By repeating this process over a period of several months these workers obtained 100 mg. of a crystalline material. We made no attempt to carry out the crystallization of the vitamin, but tested our materials as left on evaporation of the decomposed platinum precipitates.

"Torulin" is the name given by Kinnersley and Peters (21) to a B_1-containing, B_2-free yeast concentrate the preparation of which they have developed over a period of several years. In simplified form, a Kinnersley-Peters concentrate has been extensively used by English workers in place of yeast as a source of B_1 in assaying vitamin B_2 by the rat growth method. Our fourth concentrate (H_4) was made by the Chick and Roscoe (22) modification of this method. Ten lbs. of fresh brewers' yeast suspension was washed repeatedly with water, and then boiled with water containing 0.02% acetic acid. A precipitate formed on the addition of a 25% solution of lead acetate to this extract was rejected. The filtrate was treated with barium hydroxide to precipitate gummy material, and the filtrate from this precipitate brought to pH 4.0 by the addition of sulphuric acid. After removing barium sulphate, the filtrate was treated with Hopkins's reagent (10% solution of mercuric sulphate in water containing 70 cc. of sulphuric acid per liter) and the precipitate rejected. Traces of the heavy metals were removed by hydrogen sulphide, this being the step which offered the greatest difficulty. After adjusting the reaction to neutrality, the vitamin was adsorbed on norite previously boiled with dilute hydrochloric acid and washed free from acid. As in the Smith method (H_1) the vitamin was eluted with dilute acid alcohol (50 cc. alcohol, 50 cc. water, 1 cc. concentrated hydrochloric acid) and evaporated at reduced pressure to about 175 cc. of a clear, brownish yellow liquid of pleasant odor.

B. THE DEVELOPMENT OF POLYNEURITIS

The diet employed in our experiments consisted of:

Deactivated Casein	18%
Argo Corn Starch	58%
Harris Autoclaved Brewers' Yeast	10%
Olive Oil	9%
Salt Mixture (McCollum's 185)	4%
Cod-liver Oil	1%

The casein was deactivated by washing for three days in distilled water and for seven days in 0.2% acetic acid, and dried. The rats employed in this work were "Vitamin Grade" rats purchased from the Sprague and Dawley Company of Madison, Wisconsin, shipped within a day or so of weaning, and kept for two to three days on a varied diet. They were used in experimental work when their weights were between 35 and 45 grams. In our earliest work, the rats were kept in two groups of 12 and 13 rats in two cages. Of the first group of 12 rats but four developed paralytic symptoms (on the 41st, 73rd, 91st, and 101st days). Of the second group, seven became paralyzed between the 56th and 70th days. Four rats of the first and six of the second group died without developing characteristic symptoms, usually after a prolonged loss of weight at an approximate average rate of a gram daily for three or four weeks. As five of these rats died within a period of a few days it was thought that the loss of weight might have been due in part to the spread of an infection. In subsequent work the animals were kept in individual cages equipped with wire screens to limit coprophagy. In view of the work reviewed in a previous section, the large number of rats dying without developing polyneuritis might have been ascribed to a too complete exclusion of vitamin B_1 from the diet. Against this is the fact that we were much more successful in subsequent work, using either the same or an even more limited diet. Furthermore, several of the rats survived for over 150 days with weights between 120 and 150 gm. without developing the desired symptoms.

In a second experiment, 26 rats were placed in individually screened cages between the 56th and 60th days on the diet. As we had been impressed by the survival of the rats in the previous experiment, which it did not seem could be ascribed to refection, we began re-autoclaving for ourselves at 20 lbs. pressure for 4 hours in 1-inch layers the commercial autoclaved yeast, to eliminate more completely B_1. There was no instance of paralysis before the institution of these two procedures—the use of individual cages, and the re-autoclaving of the yeast. Of the 26 rats in this experiment, 23 eventually were brought into a polyneuritic condition. One of the three which failed to develop polyneuritis received an addition to the diet not pertinent to the present considerations. The first rat to show symptoms did so at 74 days; 14 were not paralyzed until after 100 days. That the onset of the symptoms occurred later than in the experiments reported by Smith (14) may have been due to the fact that the re-autoclaving of the yeast and use of individual cages was not begun until late.

In the third series, most of the twenty-six rats were placed in the individual cages after twenty days on the diet, and the use of re-autoclaved yeast was started as early as the seventeenth day. Six of the rats in this series died without paralytic symptoms after 31, 41, 43, 50, 51 and 83 days on the diet. Seventeen of the rats developed polyneuritis after 40, 45, 49, 52, 53, 54, 57, 58, 61, 62, 65, 67, 70, 85, 85, and 95 days. Only three of the rats had failed to become polyneuritic by the 139th day and two of these had received certain dietary additions to be discussed at another time. The onset of polyneuritis occurred earlier in this third series than in the preceding series. It seems that the re-autoclaving of the yeast may have been responsible for this, for a larger number of the rats died before the symptoms became evident.

In the present state of our knowledge, it seems futile to speculate further as to the exact causes which lead to the earlier or later development of the symptoms, or to the failure to develop them before death. The exact conditions of extracting the casein and autoclaving the yeast probably vary from laboratory to laboratory. Inherited characteristics in the rats used may also play a part. Nevertheless, these experiments show that the polyneuritic condition may readily be produced in young rats. Having once empirically established satisfactory conditions, it seems well to utilize them with as little variation as possible, postponing further studies of the exact degrees of deficiencies of the various B factors which are essential to their production until such factors shall have been isolated as pure compounds.

TABLE I

Assay of Preparation H_1

RAT NUMBER Series, Group, Number	WEIGHT	CONDITION	DAY ON DIET WHEN INJECTED	DOSE	RESULT	DAYS BEFORE RECURRENCE OF SYMPTOMS	WEIGHT CHANGE	DAY DOSE	WEIGHT RECOVERY TIME PER 0.01 MG.
I, I, 5	62 gm.	Cartwheel turning	107	10 mg. (approx.)	No change	—	+1 gm. first day. Lost 5 gm. in next two days	—	—
Same rat. Reinjected after 3 days	—	—	—	25 mg. approx	Died	—	—	—	—

C. THE POLYNEURITIC CONDITION

Our observations of rat beriberi agree so well with those given in our summary of Hofmeister's description (13) that we shall limit ourselves to mentioning a very few points. The onset of outstanding symptoms usually followed a rather rapid loss of weight occurring over a period of four or five days. Our observations on this point agree rather more with the weight curves of Smith (14) than with those of Hofmeister (13). We have noted as a rather constant finding in the otherwise somewhat variable picture, that the rats grasp the screens of their cages very firmly with the fore and hind claws, so that they are removed for examination with difficulty. We have noted head retraction in only a few rats, the occurrence of spastic paralysis of the hind legs being the most frequent finding. Rapid meaningless waving of the head, twitches, and circle walking also occurred, but rather less frequently. Many exhibited convulsions when stimulated, or particularly, when held upon their backs. Such rats often rolled for considerable distances. The occurrence of such varied symptoms among rats on the same diet speaks against an attempt to assign particular features of the picture to one or another factor as B_1 or B_1. Our concentrates in all probability contain both of these factors, as judged by a comparison of the methods of preparation with those of the Oxford workers, but we have made no attempt in assaying them to distinguish between these factors, or to evaluate each separately.

In none of our rats did we observe darkening of the tail or lesions about the eyes, ears or nose—symptoms of a B_2 deficiency (23). It is also rather interesting that we have not seen the red, swollen paws considered by some (19) a result of a B_1 deficiency and by others, as Leader (23), and Sherman and Sandels (24), included among the manifestations of a lack of B_2.

D THE EVALUATION OF THE CONCENTRATES

In Tables I, II, III are given the results of our tests of concentrates H_1, H_2, and H_3, respectively. All of the injections were made subcutaneously. Our records of the exact con-

TABLE II

Assay of Preparation H_2

RAT NUMBER Series, Group, Number	WEIGHT	CONDITION	DAY ON DIET WHEN INJECTED	DOSE	RESULT	DAYS BEFORE RECURRENCE OF SYMPTOMS	WEIGHT CHANGE	DAY DOSE	WEIGHT RECOVERY TIME PER 0.01 MG.
II, II, 2*	55 gm.	Moderate	95	0 05 mg.	Recovery by next day	33	+16 gm. in 17 days	1 51γ	6.5 days
II, II, 8*	74	Severe	98	0 05	Very slight improvement by next day. Practically complete recovery by second day	5	+5 gm., but this did not occur until after first day	10γ	Died before again reaching injection weight
II, II, 9	46	Severe	88	0 05	Improved next day	9	+5 gm.	5 5γ	1.6 days +
Same—Re-injected	39	Severe	98	0 03	Slight improvement next day, but no complete cure	—	+5 gm. in 4 days	—	Died before again reaching injection weight

*These rats had been receiving isobarbituric acid as a dietary addition in other experimental work.

TABLE II—(*Continued*)

RAT NUMBER Series, Group, Number	WEIGHT	CONDITION	DAY ON DIET WHEN INJECTED	DOSE	RESULT	DAYS BEFORE RECURRENCE OF SYMPTOMS	WEIGHT CHANGE	DAY DOSE	WEIGHT RECOVERY TIME PER 0.01 MG.
III, I, 4	38 gm.	Moderate	40	Unknown (x)	Improved on next day. Cured by second day	54	+49 gm. in 19 days	—	$\frac{71 \text{ days}}{x}$ with polyneuritis recurrence intervening
III, I, 9*	52	Moribund	47	0 15 mg.	Died in a few hours	—	—	—	—
III, II, 3	52	?	63	0 04	Improved. 3 days before all symptoms relieved	7	+3 gm.	5 7γ	2.0 days
III, II, 6	44	Moribund	50	0 12	Died in a few hours	—	—	—	—
III, II, 9	44	Moderate?	50	0 05	Improved by next day, but a slight dragging of left hind leg persisted 4 days	7	+4 gms.	7 1γ	1.6 days
III, II, 11	45	Moribund	61	Unknown	Died in a few hours	—	—	—	—

*These rats had been receiving isobarbituric acid as a dietary addition in other experimental work.

ditions of the animals in the earlier experiments were not as complete as we should now desire, as we were not then familiar with the symptoms.

Only two tests were made with preparation H_1 (Table I), as it was found by another worker in this laboratory that the commercial dry yeast used as a source of this preparation was unsatisfactory as a source of vitamin B for rats used in experiments on vitamin A. While not enough experiments were made to eliminate a false negative result, it was felt that in view of the large doses used, the preparation could have had comparatively little activity. The doses used are not accurately known as in attempts to determine total solids there was always some charring. Evaluation of the concentrates on a basis of total solids was desired for the determination of extinction coefficients in the following paper.

From the results in Table II it is apparent that H_2 was a concentrate of great antineuritic potency, approaching if not equalling the most active preparations described by A. Seidell (25) which were, however, prepared by a somewhat different technique. Higher potencies have been reported by Guha and Drummond (26) (5γ = pigeon day dose) and recently, by Windaus (27) (2.4γ = pigeon day dose). Disregarding the three moribund rats, which died shortly after injection, cures are seen to have resulted in six cases in which injections of 0.05 mg. or less were used. With 0.05 mg. there were no failures. The results were somewhat less decisive in the cases in which 0.03 and 0.04 mg. were used. The minimal curative dose of this preparation was certainly less than 0.05 mg., and possibly less than 0.04 mg. When border-line doses were used, the convulsions were promptly cured, and usually also severe degrees of spastic paralysis, but slight degrees of lameness and incoördination persisted two or three days longer. The gain in weight did not begin until the second day in such cases. In view of the work of Miss Reader (19) it might be thought that in such cases the limiting factor was B_4 and not B_1. It is very probable that this preparation contains both of these factors, as the Jansen-Donath crystals (although pre-

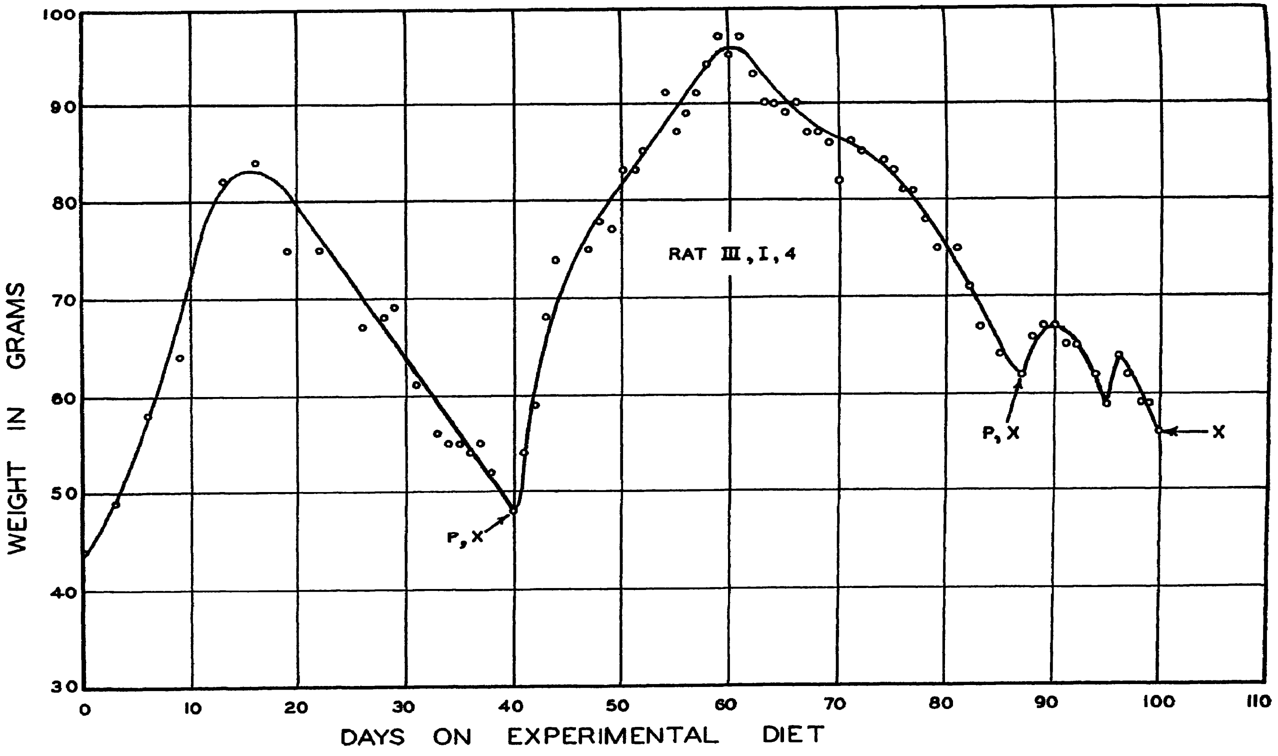

Fig. 1—Weight Curve of Rat (III, I, 4), Injected with Preparation H_2.
"P" denotes polyneuritis. "X" denotes injection of H_2.

pared from rice polishings) have been shown by the English workers to contain both. We feel, however, that in so speculating, we should be on rather dangerous ground, and merely prefer to state the activity as 0.04 – 0.05 mg., irrespective of whether B_1, B_4, or a mixture is the limiting factor or factors. Expressed as the day dose, our results are, as might be expected, much more variable. The five values obtained varied between 1.5 and 10γ, with a mean value of 6.0γ. The mean "recovery time" that is, the time required for the weight to return to its value at the time of injection per 0.01 mg., was 2.9 days. The four individual values were 6.5, 1.6, 2.0, 1.6 days. This could also be expressed as a weight day dose (3.4γ) by dividing the number of days of excess weight into 0.01 mg.

In examining this concentrate a preliminary injection of a small amount of the material was made to get some idea of the activity of the preparation, so that animals and materials could be saved by not using too small amounts if the activity proved to be low. We reproduce this curve (Fig. 1) as an indication of the prolonged effect of a rather large amount of highly active concentrate. A gain of 49 gms. occurred in the 19 days following the single injection, which served to protect the rat from a recurrence for nearly two months. The initial paralysis of this rat was, however, only moderate. This long period of protection indicates a very considerable capacity of the rat to store antipolyneuritic factors. Fig. 2 illustrates the use of repeated recurrences and injections, an entirely feasible procedure if used with caution. (Rats II, II, 9, and III, II, 9.)

Two of the 19 injections used for H_3 (Table III) must be eliminated from consideration as the rats died within a day of the injection. The least dose which was found to produce a definite cure was 0.10 mg.; the rat which received it was but slightly paralyzed when injected. 0.12 mg. produced an incomplete cure, which lasted but two days. In one case 0.16 mg. had no effect, and in another resulted in an incomplete cure. 0.18 mg. was effective in the three cases tried. 0.20 mg. varied, failing in two attempts to cure recurrences of symptoms,

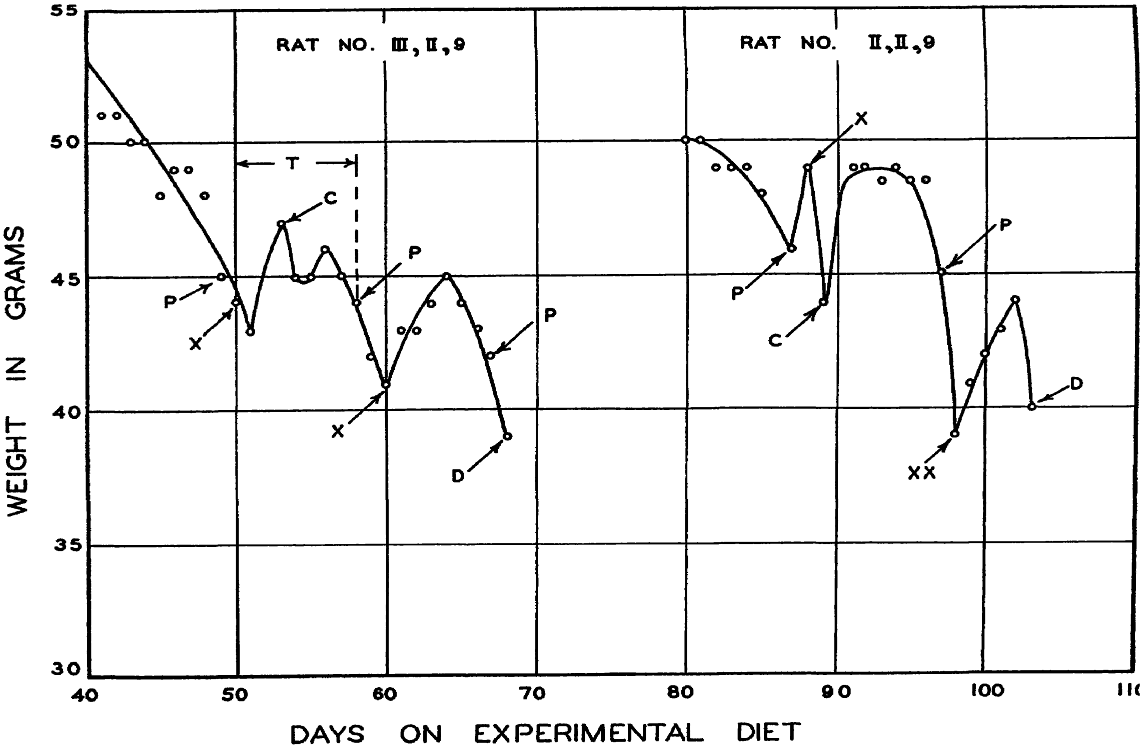

Fig. 2—Weight Curves of Rats (II, II, 9) and (III, II, 9), Injected with Preparation H_2. "P" denotes polyneuritis. "X" denotes injection of 0.05 mg. H_2. "XX" denotes injection of 0.03 mg. H_2. "T" denotes weight restoration time. "C" denotes cure. "D" denotes death.

TABLE III

Assay of Preparation H_3

Rat Number Series, Group, Number	Weight	Condition	Day on Diet When Injected	Dose	Result	Days Before Recurrence of Symptoms	Weight Change	Day Dose	Weight Recovery Time Per 0.01 mg.
II, II, 1	69 gm.	Severe	142	0 16 mg.	Died within 2 hours	—	—	—	—
II, II, 7	73	Slight, but definite leg spasticity	126	0 10	Cured within 2 days	18	+7 gm. in 2 days after which remained nearly constant 2 weeks	5 6γ	1.60 days
II, II, 12	72	Severe	140	0 08	Only very slight improvement. Died after 3.5 days	—	+1 gm.	—	—
III, I, 7	46	Severe	85	0 10	Died same day	—	—	—	—
III, II, 3	52	Slight	72	Unknown (y)	Cured next day	24	+20 g. in 5 days	—	$\frac{32 \text{ days}}{y}$
III, II, 12	55	Moderate	71	Unknown	Cured next day	2	+4 gms.—died suddenly on the third day	—	—
III, II, 13	53	Severe	87	0 10	Slight improvement, became able to walk, but weight loss continued. Died in 4 days	—	−5 gm.	—	—

TABLE III—(Continued)

RAT NUMBER Series, Group, Number	WEIGHT	CONDITION	DAY ON DIET WHEN INJECTED	DOSE	RESULT	DAYS BEFORE RECURRENCE OF SYMPTOMS	WEIGHT CHANGE	DAY DOSE	WEIGHT RECOVERY TIME PER 0.01 MG.
II, I, 3	80 gm.	Moderate	149	0 18 mg.	After 1 day, only a slight lameness remained; persisted 4 days	38	+35 gm.	4.7γ	2.30 days
II, I, 6	60	Moderate	148	0 16	No change in 2 days	—	−3 gm.	—	—
Reinjected	57	Moderate	150	0 18	Improved next day, cured on second day	4	+6 gm.	45γ	0.33 days
Reinjected	55	Moderate	157	0 20	No improvement	—	+4 gm.	—	0.10 days
Reinjected	55	Moderate	159	0 20	Improved in 2 days	4	+3 gm.	50γ	0.18 days
II, I, 13	69	Slight to moderate	145	0 12	Cured next day, but died after 3 days	2	−7 gms.	60γ	—
II, II, 3	61	Fair	151	0 20	Improved next day. Very slight lameness continued 3 days	4	+2 gms.	50γ	0.18 days
Reinjected	59	Moderate	155	0 24	Improved greatly on second day. Died suddenly on fourth day	3	Maintained weight 2 days, then loss	—	—

TABLE III—(*Concluded*)

RAT NUMBER Series, Group, Number	WEIGHT	CONDITION	DAY ON DIET WHEN INJECTED	DOSE	RESULT	DAYS BEFORE RECURRENCE OF SYMPTOMS	WEIGHT CHANGE	DAY DOSE	WEIGHT RECOVERY TIME PER 0.01 MG.
III, I, 4	59 gm.	Moderate	95	0.16 mg.	Marked improvement on first day then gradually became slightly worse	5 (for pronounced symptoms to recur)	+5 gms. on first day	32γ	0.19 days
Reinjected	56	Moderate	101	0.18	Cured except for very slight leg weakness on first days	6	+2 gms.	30γ	0.19 days
Reinjected	50	Moderate Circus movements and incoordination were most marked symptoms	107	0.20	Some improvement after 3 days	—	+1 gm.	—	0.15 days
IV, I, 3	39	Moderate	48	0.20	Complete cure next day	31	+24 gms. in 17 days	6.4γ	Polyneuritis recurred before weight returned to that at injection

but succeeding in three trials. 0.24 mg. was successful in a treatment of a recurrence in so far as the polyneuritic symptoms were concerned, but a gain in weight was not noted, and the rat died suddenly from an undetermined cause after four days. These experiments were made with a stock solution of the concentrate which was kept in a refrigerator during the somewhat extended period of the tests. That a deterioration of the material occurred is excluded by the fact that the last injection produced one of the most pronounced effects in the entire series. It seems worthy of comment that the instances of unsatisfactory response with large doses were obtained in rats which had had previous attacks of polyneuritis either cured by injections of a sufficient amount, or in which an insufficient dose had previously been given. Our experience is that when recurrences of polyneuritis are treated, the results may not always be the same as when the first attacks are treated. Especial caution is always necessary in evaluating dosages in such instances. Sebrell and Elvove (15), it may be recalled, used the sixth, and in one instance the eleventh recurrence, while Hofmeister (13) believed improvement could not be noted after the second recurrence. From the results cited in Table III, with due allowance for the inherent variations involved in all biological tests, it seems that the minimal curative dose of H_1 can safely be placed at 0.16–0.20 mg.

The values found for the day dose were very much more erratic, varying from 5 to 60γ with a mean value of 32γ. It seems obvious that this method of stating the results should not be employed unless a large number of experiments is made. The "weight recovery time" in days per 0.01 mg. showed better agreement, the values being 1.60, 2.30, 0.33, 0.10, 0.18, 0.18, 0.19, 0.19, 0.15. The mean is 0.58. Expressed as the dose per day required to maintain weight this would be $\frac{0\ 01}{0.58}\text{ mg.}\times 1000 = 17\ 2\gamma$.

The preparation shown in Table IV was furnished us through the kindness of Dr. L. R. Cerecedo, who prepared it from rice polishings by the Jansen-Donath method, using Jansen's modification (28) in which cadmium chloride re-

TABLE IV

Assay of Preparation A III of Cerecedo

RAT NUMBER Series, Group, Number	WEIGHT	CONDITION	DAY ON DIET WHEN INJECTED	DOSE	RESULT	DAYS BEFORE RECURRENCE OF SYMPTOMS	WEIGHT CHANGE	DAY DOSE	WEIGHT RECOVERY TIME PER 0.01 MG.
II, I, 4	101 gm.	Moderate	114	0.96 mg.	Improved on next, and practically cured on the following day	16	+13 gms. in 11 days	60γ	0 15
II, I, 7	50	?	110	0 15	Died after 1 day. No cure	—	−3 gms.	—	—
II, I, 11	44	?	109	1 26	Died in a few hours	—	—	—	—
II, II, 5*	60	?	105	0 05	Lived 4 days, with steadily progressing symptoms	—	−8 gms.	—	—
II, II, 11	49	?	103	0 05	Died in a few hours	—	—	—	—
II, II, 13	55	?	106	0 05	Died in 1 day	—	—	—	—
III, II, 2	41	?	58	0 08	Died after 1 day	—	−1 gm.	—	—
II, I, 2	64	Moderate	72	0 30	Died after 2 days, with steadily progressing symptoms	—	−6 gms.	—	—
II, I, 6	53	Moderate (a 3rd recurrence)	74	0 50	Died the next day without improvement	—	—	—	—
IV, II, 3	38	Slight	53	0 50 (approx.)	Died the next day. No improvement	—	—	—	—

*This rat had received, as part of another investigation, isobarbituric acid in its diet for several days before the onset of symptoms.

places platinum chloride as a precipitant. Freudenberg and Cerecedo (29) stated that the curative and growth-promoting dose of this preparation probably lies between 0.010 and 0.025 mg. As their tests were made upon mice, which according to Bing and Mendel (30) require about half as much B_1 as do rats, we made a series of tests upon rats so that a direct comparison of this preparation with our concentrates would be possible.

While it is true that a few of these ten rats were probably too severely affected for any cure to have been expected, this was by no means always the case, as several lived for two to four days after the injection. In but one case was a cure or an improvement noted, and then only with the rather large dose of 0.96 mg. The material was exhausted before further tests could be made, but it is evident that the preparation possesses but slight antineuritic activity. Further, rat III, I, 5, which had for some time previously maintained a nearly constant weight of 42 gms. but which had not developed polyneuritis, was injected with 0.05 mg. of the concentrate. No effect could be noted. Three days later 0.20 mg. was injected, and again the weight of the rat was unaffected. About ten days later, the weight began to decline, and a week later the rat died. These results are quite unlike those shown by Freudenberg and Cerecedo (29) in a series of nine mouse-weight curves.

A possible cause of the discrepancy is to be found in the nature of the symptoms described by these authors. They speak only of "appreciable loss in weight, the arched back, and darkening of the tail". These symptoms in the paper of Bing and Mendel (30) to which they refer are described and pictured as among those of a B_2 deficiency, while young mice deprived of vitamin B_1, are said to die within three weeks. The symptoms the alleviation of which Freudenberg and Cerecedo studied were produced from the 25th to 35th *days of age* of the mice, which were placed upon the experimental diet at the age of 21 days. A maximum of 14 days upon the experimental diet seems an unusually short time for the production of true polyneuritis in young animals.

Attempts to determine the total solids in the H_4 preparation (Peters' concentrate) solution failed as evaporation in a vacuum desiccator resulted only in the formation of a viscous brownish-black deposit. For this reason, quantitative determinations of the minimal curative dose could not be undertaken. We have, however, found that the administration of small amounts of this liquid by means of a pipette indicates it to possess a very considerable curative and weight-promoting activity. In but one of five rats tested did a complete cure fail. In the others, in which a small amount was given daily, there was a cure of the convulsive and extreme paralytic symptoms in one to two days. The residual weakness persisted a few days longer. One of these rats, very severely paralyzed when the treatment was started, retained for over a week a state of great excitement, while the weight increased rapidly. The excitement eventually subsided, and a complete cure resulted. This concentrate, given in amounts probably considerably in excess of the minimal doses, had, as indicated in Fig. 3, a pronounced growth-promoting effect. It may be recalled that the English workers stated that preparations made by this method contain both B_1 and B_4.

DISCUSSION

Agreement by all who use the method as to the exact symptoms which should be exhibited at the time the injection is made is a prerequisite to the development of the method to a point at which the findings of various workers may be reasonably compared. It could scarcely be expected that the least dose capable of curing a rat exhibiting only a slight impairment of the use of one leg should be effective in improving the condition of a rat showing cartwheel turning. Furthermore, the minimal dose required to produce a cure in a rat developing the latter symptom rather suddenly might be expected to be less than that required for one which had approached it over a longer period. A dose of 0.1 mg. of one of our concentrates produced in one day a cure of a rat showing only a limited spasticity of the left hind leg. This was accompanied by an

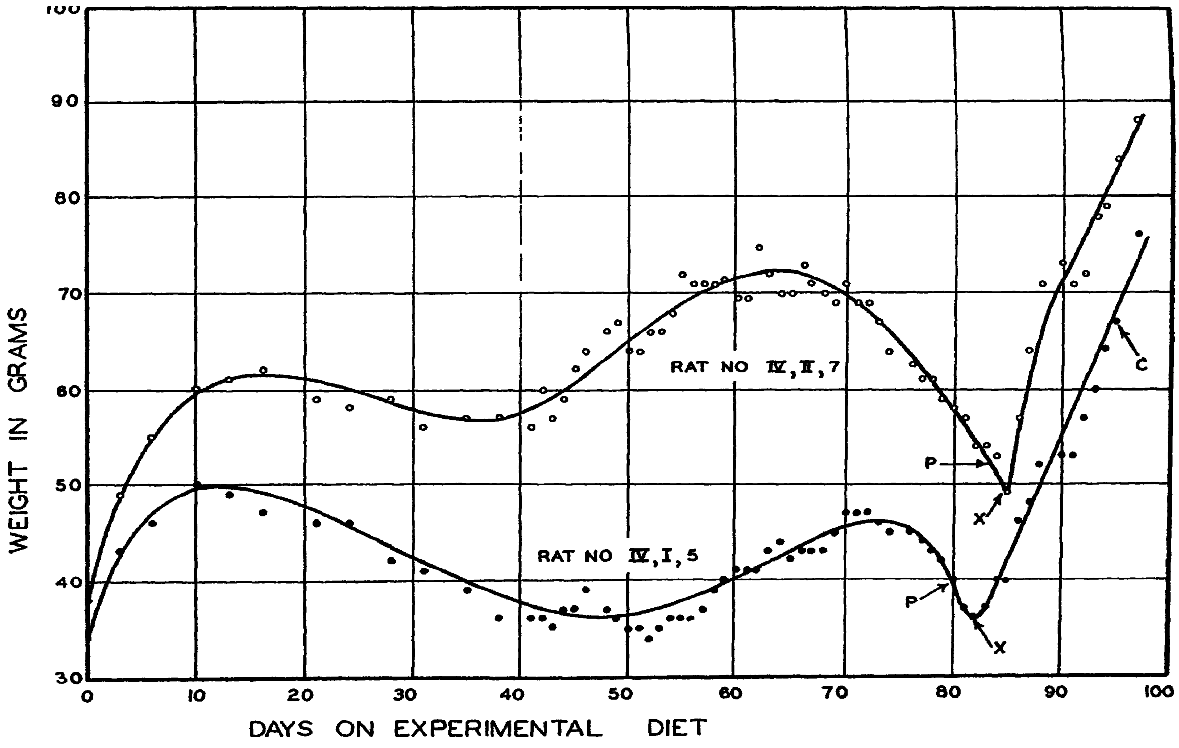

Fig. 3—Weight Curves of Rats (IV, II, 7) and (IV, I, 5), Fed Preparation H_1. "P" denotes polyneuritis. H_4 given daily from "X". Gradual improvement from "X" to cure at "C". For a discussion of the early minima on these curves, see a forthcoming paper, to appear shortly in this journal.

increased appetite and a gain of 7 grams in two days, the symptoms recurring only after 18 days. The same dose given to a rat exhibiting an extreme degree of cartwheel turning produced a slight improvement by the next day, and enabled the rat to walk, although poorly, by the second day. The weight, however, merely remained constant for one day, after which a gradual loss of weight ensued, and the rat died four days after the injection.

Should one adopt slight degrees of paralysis, as suitable for injection, there would always be an uncertainty involved. The more severely affected animals frequently exhibit much more striking effects. But in their use there is the danger of false negative results unless many animals are used, for, either the brain lesions described by Hofmeister (8) may have progressed to such an extent as to be irremediable, or shock may be induced by the injection, leading to death within a few hours. Probably the best course is to use animals so definitely affected as to be unable immediately to recover themselves when held on their backs for a few moments, and to exercise considerable critical judgment in interpreting the results. In this we agree with Sebrell and Elvove (15). As frequently but very small quantities of the material to be tested are available, large numbers of injections may be impossible; it is therefore necessary to select with care animals in the most suitable condition to be tested. Animals flaccidly paralyzed, inert, and rather cold should never be used. In interpreting the results, three methods have been suggested: (a) the weight recovery time by Hofmeister (13), (b) the minimal curative dose by Smith (14), and (c) the day dose (the dose divided by the number of days of cure) by the English workers (18). We present our results in terms of each of these methods (Table V). The order of activity of the concentrates tested remains the same whichever method is adopted. The agreement between the individual tests varies most in the case of the day dose (Peters), probably because of the difficulty of determining (again) the exact time of recurrence of the symptoms which may start very gradually.

The results of the earlier workers with pigeon polyneuritis were frequently marred by the occurrence of spontaneous cures, or cures obtained under the influence of heat, distilled water, glucose, or various other compounds. Smith (14) claimed much greater specificity for the rat method, but made no mention of the occurrence of spontaneous cures, nor have we found any other reference to it. We have, however, observed spontaneous cures in two of forty-four paralyzed rats, but in these animals only the earliest symptoms had been evident. In neither would we have considered the condition as at all suitable for injection. The danger of the introduction of error from this source appears negligible, being practically eliminated by the use of several rats in fixing the minimal curative dose of a given concentrate.

TABLE V

Comparison of Different Concentrates.

CONCENTRATE	MINIMAL CURATIVE DOSE	DAY DOSE AS USED BY PETERS	WEIGHT DAY DOSE (Modified from Hofmeister)
H_1	?	?	?
H_2	0.04 – 0.05 mg.	6.0γ	3 4γ
H_3	0.16 – 0.20 mg.	32γ	17 2γ
A III of Cerecedo	0.5 – 0.96 mg. ?	60γ ?	67γ ?

There is a satisfactory agreement in the ratio of activities by the first two methods, which depend upon the antineuritic symptoms. This indicates that even the day dose method, despite the extreme variations between the individual animals, gives results not greatly in error, providing only that sufficient tests be made. There is, however, much less likelihood of error in utilizing the minimal curative dose, when limited numbers of animals are employed.

The ratios between day doses, in the last column, based on weight recovery, agree very well with those in the previous column which depend upon the recurrence of polyneuritis. This agreement may be fortuitous, depending upon the presence of B_4 in the concentrates. The English workers hold that B_1 does not produce an increase in weight in the absence of B_4. Until the effect of pure B_1 upon the weight has been conclusively determined, methods based upon the cure of polyneuritic symptoms are to be preferred.

CONCLUSIONS

From our experience in the assaying of these concentrates, we recommend the following procedure:

1. Young rats of 35 to 45 gms. weight should be used.

2. They should be kept in individual cages, with screened bottoms to limit coprophagy, and supplied with a plentiful amount of the diet prescribed on page 11.

3. The symptoms arising after the fiftieth day should be carefully observed in several rats, and no attempt should be made to assay any concentrates until one has become familiar with the variations in symptoms exhibited by different individuals. Animals are not ready for the assay when they exhibit mere loss of weight or limitation in the use of one or both hind legs. One should wait until convulsions or rolling may be elicited by holding the animal upon its back. The concentrate tested should be injected subcutaneously before this state has persisted for more than a day to avoid false negative results. Flaccidly paralyzed, inert, or rather cool rats are unsuitable.

4. A series of graded doses of the preparation should be given to about five rats to determine the approximate curative dose. Within one, or at most two, days of the injection of a curative dose, the animal should be able to right itself easily when held on its back, and should exhibit no convulsive symptoms. Any loss of weight should have ceased, and the improvement in the use of the legs should be marked, although not necessarily complete. A cure should persist at least three days before the symptoms recur.

5. The approximate minimal curative dose thus found should then be confirmed by a series of slightly lesser and slightly greater doses on about five rats each.

6. Animals with recurrent attacks of polyneuritis may be employed, but their use is not advised for accurate quantitative results unless many are employed.

7. The danger of error due to spontaneous cures is remote.

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DEDUCTIONS AS TO THE CHEMICAL CONSTITUTION OF VITAMIN B_1 FROM THE ABSORPTION SPECTRA OF B_1 CONCENTRATES

By FRANCIS F. HEYROTH AND JOHN R. LOOFBOUROW

SUMMARY

Parallel spectrographic and biologic data regarding eight vitamin B_1 concentrates are presented. From a comparison of the absorption spectra and the biological activities, it is concluded: (1) that biologically inactive purines or pyrimidines account for a large part of the absorption of the less active concentrates; (2) that vitamin B_1 itself is characterized by an absorption maximum at 2600 Å.; and (3) that vitamin B_1 is indicated by its spectrographic properties to be a pyrimidine-ring-containing compound or a compound of the same type as ergothioneine.

INTRODUCTION

For some time we have been actively interested in the ultra-violet absorption spectrum of vitamin B_1 from the standpoint of the information it might yield as to the chemical nature of the vitamin.

We were led to undertake an investigation of B_1 spectra through our study of the ultra-violet absorption of purines and pyrimidines (1), the sources of which are similar to those of B_1 concentrates. In a previous paper (2), we pointed out similarities in the absorption of three B_1 concentrates to the absorption of purines and pyrimidines, the distinguishing feature of which is a pronounced maximum at 2600 Å. Two of the concentrates studied had also considerable absorption in the short-wave ultra-violet (near 2300 Å.).

These investigations led us to conclude that vitamin B_1 might be characterized either by a maximum at 2600 Å., or by strong absorption in the short-wave ultra-violet. Because the concentrates studied were admittedly impure, however, we did not feel warranted at that time in definitely designating either of these two types of absorption as characteristic of B_1. We preferred to undertake first parallel spectrographic and biological tests of various B_1 concentrates, a method of approach apparently not employed for this vitamin by other workers up to this time.

As a result of these investigations, with which we have been occupied for the past year, we have accumulated sufficient evidence to say that in our opinion the 2600 Å. maximum is characteristic of B_1. This conclusion gives us a basis for deductions as to the possible chemical structure of the vitamin.

Since the preparation of our previous report, Guha (4) has published curves indicating two B_1 preparations to absorb in the 2600 Å. region (without, however, designating this absorption as characteristic of the biological activity), and Windaus et al. (5) have obtained a crystalline preparation, having a pronounced absorption maximum at 2600 Å., which they believe to be the pure vitamin. These results offer considerable confirmation of our opinion that vitamin B_1 is characterized by a 2600 Å. band.

In the following pages, we consider the correlation of the absorption and biological activity of four preparations made in this laboratory, of three kindly supplied by other workers, and of the Windaus preparation as described in the literature, and discuss the possible chemical implications of the conclusion therefrom.

EXPERIMENTAL

The methods of preparation and biological assay of our own concentrates have been described in the preceding paper (3).

The selection of the dose representing the activity of the Windaus preparation at first appeared to offer considerable difficulty because, while the activities of all the other preparations were known in terms of the minimal curative rat doses, Windaus gave the curative day dose for pigeons. His value for this dose was 2.4γ. A simple way of converting this value to the minimal rat curative dose is, however, afforded by the following considerations. Windaus stated that he followed exactly the technique by which Jansen, Kinnersley, Peters, and Reader (6) assayed the Jansen-Donath crystals and found 7-9γ as the pigeon curative day dose. This means that the

Windaus preparation is, if both of these assays may be assumed to be correct, 8/2.4, or 3.33 times as active as the Jansen-Donath crystals. A sample of the latter crystalline preparation (J.-D.) has also been assayed by M. I. Smith (7) by the method which we employed (3) in the case of preparations H_2 and H_3 and by which Smith obtained the values used in the case of the Seidell preparations. As Smith found 0.04 mg. for the minimal curative rat dose of the Jansen-Donath crystals, the probable minimal curative rat dose of the Windaus crystals is 0.04 /3.33, or 0.012 mg.[1]

The ultra-violet absorption spectra were obtained by a photographic method recently described by us (2). In most cases, aqueous solutions were used, convenient concentrations for determining the complete spectral curve being chosen in each instance. The concentrations employed in the measurements are given in the figures.

The absorption spectra of all the concentrates measured, together with three curves from the recent literature [Guha (4) and Windaus (5)], are shown in Fig. 1. These have all been reduced to the same basis of concentration and cell

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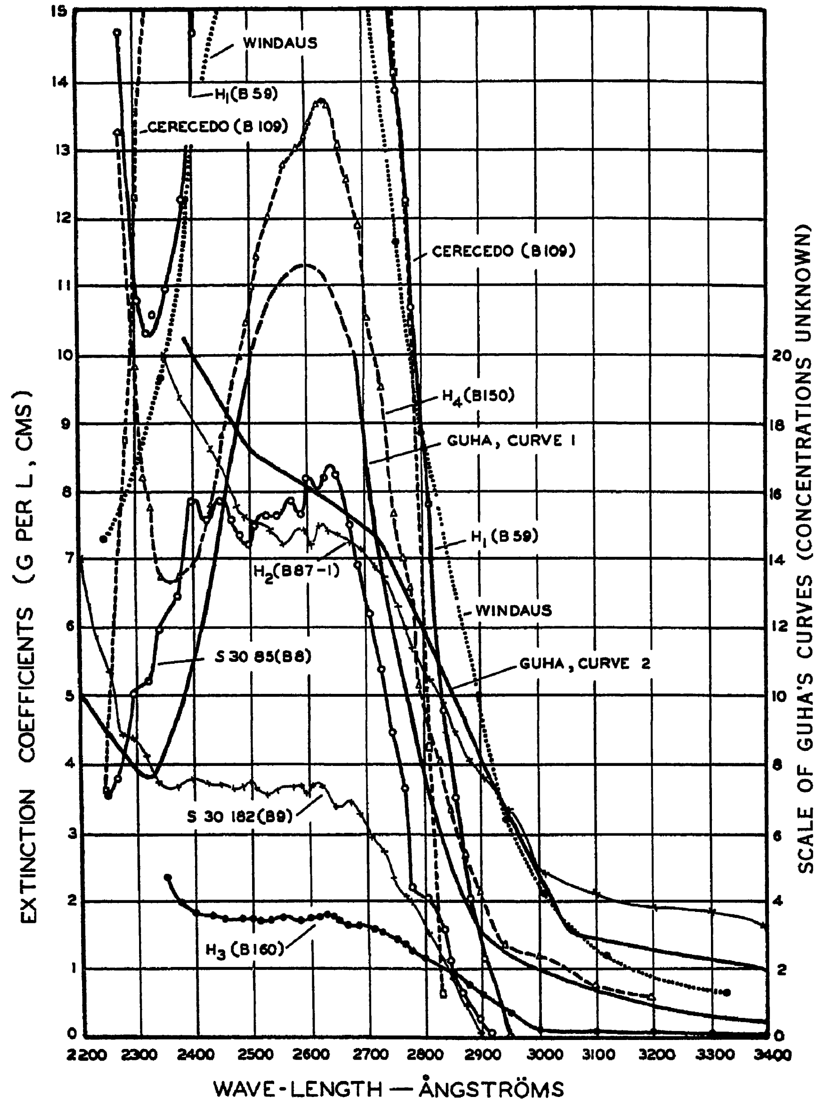
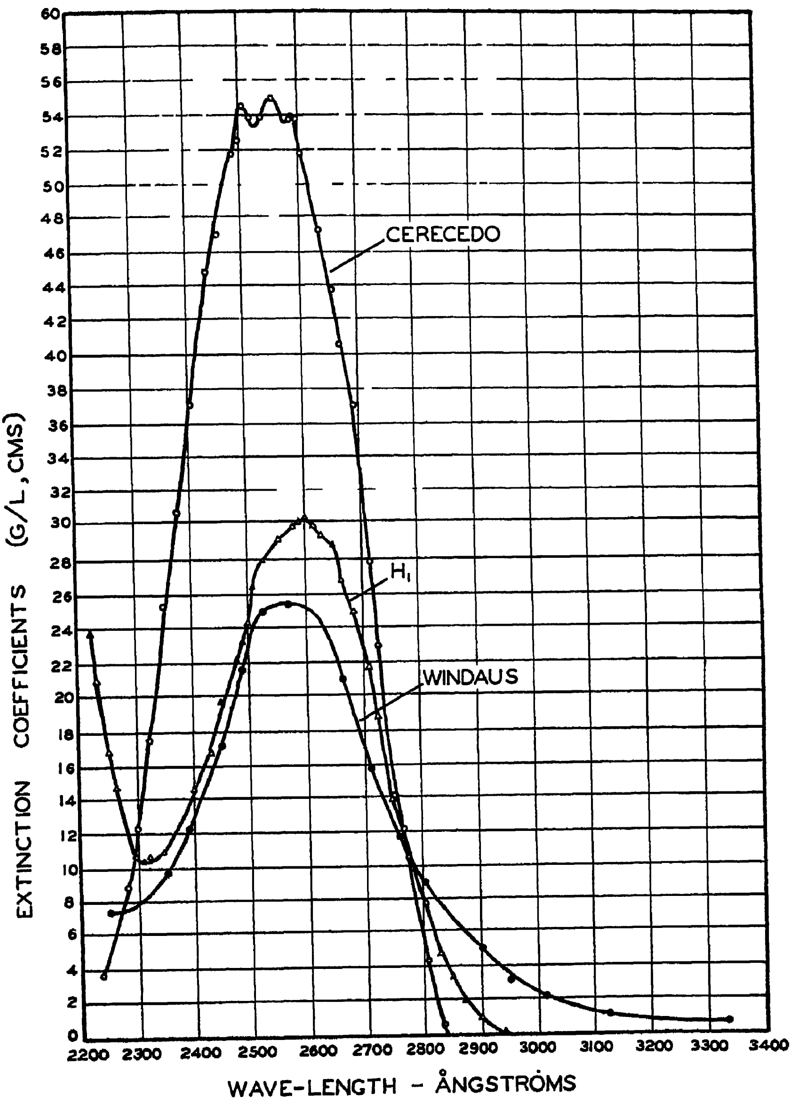
[1] Van Veen (8) has recently stated that the dose of a sample of the Windaus preparation is identical with that of his crystals, which in turn have the same potency as the Jansen-Donath crystals. He used a much less satisfactory minimal protective dose assay on rice-birds, there being a common supply of food containing the vitamin preparations for ten birds. Against the identity of the van Veen and Windaus crystals is the fact that the latter were readily soluble in ethyl alcohol, while the former were difficultly soluble. Van Veen admits the Windaus preparation to be purer than the original Jansen-Donath crystals since it fails to yield a precipitate with mercury sulphate and sulphuric acid, with which the Jansen-Donath crystals yield a precipitate. Further, the Windaus preparation, like the Seidell preparations and unlike the Jansen-Donath crystals, yields no Pauly reaction. Other evidence that the Jansen-Donath crystals cannot possess the highest activity possible for a pure compound is the fact that Seidell (9) has described a preparation for which the minimal curative rat dose is only 0.03 mg., which is three-fourths that of the Jansen-Donath crystals. So far as we have been able to determine, the van Veen crystals have not been assayed by one of the curative methods.



Concentrations employed in taking the spectra Cerecedo, 10 mg. per L, H_1, 25 mg per L, Seidell 30 85, 75 mg per L, H_2 and Seidell 30 182, 100 mg per L., H_3, 400 mg per L., H_4, unknown, all 2 cm cells

Fig. 1—Ultra-Violet Absorption Spectra of Various Vitamin B Concentrates



Concentrations employed in taking the spectra Cerecedo, 10 mg per L, H_1, 25 mg per L, 2 cm cells.

Fig. 2—Ultra-Violet Absorption Spectra of Concentrates Having Marked Extinction.

TABLE I

Source, Preparation, Potency, and Extinction Coefficients of Various Vitamin B_1 Concentrates.

CONCENTRATE	SOURCE	METHOD OF PREPARATION	BIOLOGICAL ASSAY[1]		EXTINCTION COEFFICIENTS (1 GM. PER L., 1 CM.)							
			THIS LAB.	OTHER LABS.	$\lambda=2250$	$\lambda=2300$	$\lambda=2400$	$\lambda=2500$	$\lambda=2600$	$\lambda=2700$	$\lambda=2800$	$\lambda=2900$
Windaus	Yeast	See (5)	—	0.012 mg.	Exterpolated 7 0	Exterpolated 8 0	13 0	23 0	25 0	16 6	9 0	5 0
Heyroth (H_2)	Fresh Brewers' Yeast	Modified Method of Jansen and Donath (3)	0.04 to 0.05 mg.	—	Exterpolated 12 5	Exterpolated 11 0	9 0	7 5	7 2	7 0	5 3	3 8
Seidell 30:85	Yeast	See (7)	—	0.05 mg.	Exterpolated 3 5	Exterpolated 5 0	7 5	7 3	8 2	6 5	2 0	0 3
Heyroth (H_3)	Fresh Brewers' Yeast	Modified Method of Jansen and Donath (3)	0.16 to 0.20 mg.	—	Exterpolated 5 0	Exterpolated 3 3	1 8	1 7	1 75	1 6	1 1	0 65
Seidell 30:182	Yeast	See (7)	—	0.25 mg.	5 5	4 3	3 8	3 7	3 6	3 1	1 7	0

[1] Rat curative dose except where otherwise stated.

TABLE I—(*Continued*).

CONCENTRATE	SOURCE	METHOD OF PREPARATION	BIOLOGICAL ASSAY		EXTINCTION COEFFICIENTS (1 GM. PER L., 1 CM.)							
			THIS LAB.	OTHER LABS.	$\lambda = 2250$	$\lambda = 2300$	$\lambda = 2400$	$\lambda = 2500$	$\lambda = 2600$	$\lambda = 2700$	$\lambda = 2800$	$\lambda = 2900$
Cerecedo	Rice Polishings	See (10)	0.5—0.96 not accurately determined	0.25 mg. Mouse *day* dose[2]	5 0	12 2	37 0	54 5	52 0	33 0	5 0	0
Heyroth (H_1)	Commercial Dried Yeast	Method of M. I. Smith See (3)	Probably inactive	—	18 0	10 7	14 7	24 4	30 6	23 6	5 0	0
Heyroth (H_1)	Fresh Brewers' Yeast	Chick and Roscoe Modification of Kinnersley-Peters Method (3)	Active—Dose not determined[3]	—	Concentration could not be determined. Quantitative extinction values could not be calculated.							
Guha Preparations	—	See (4)	—	Quantitative dose not given	Concentration not given—quantitative extinction values could not be calculated.							

[2] Equivalent to about 0.5 mg. rat curative dose.

[3] The solution was highly active, but the weight of dry matter present could not be determined.

thickness to facilitate comparison,[1] except in the case of the curves from Guha's paper (4) in which instance insufficient data were given by the author to enable us to recalculate his curves on this basis. Guha's curves have, therefore, been replotted on his own scale; no attempt should be made to compare them quantitatively with the others.

Three of the preparations (H_1), Windaus (5), and Cerecedo (10) had too great extinction values for the entire curves to be included on the scale of Fig. 1. These have been replotted on a more condensed scale to show the complete curves in the region of maximum absorption, and are reproduced on this basis in Fig. 2.

Table I shows the methods of preparation and biological potency of each of the samples, together with the extinction coefficients at eight wave-lengths in the ultra-violet spectrum as determined from the curves of Figs. 1 and 2. The preparations are listed in the order of their biological potencies.

DISCUSSION

THE PRESENCE OF PYRIMIDINES AS IMPURITIES

Our early investigations indicated that pyrimidines and (at certain pH values) purines are characterized by a marked absorption band at or near 2600 Å. (1). Later studies indicated certain vitamin B concentrates to have similar absorption (2). Because of the sources and methods of preparation, one might expect a part, at least, of this 2600 Å. absorption in the concentrates to be due to the presence of biologically inactive pyrimidine or purine impurities. Van Veen (11), indeed, has identified several purines and has isolated cytosine and uracil in late stages of the preparation of crystalline B_1 concentrates by the Jansen-Donath method. These purines and pyrimidines are common to the sources from which the concentrates are prepared, and could easily be present to a considerable extent in the final preparations. They are generally accepted to be antineuritically inactive.

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[1] This assumes that Beer's law holds for these materials.

In view of the possible presence of these highly-absorbing but inactive impurities, it is not surprising that the two least active of the preparations listed in Table I (H_1 and Cerecedo) had the most pronounced maxima at 2600 Å. H_1, for example, had been subjected to the least elaborate purification of our own preparations.

In any investigation of the absorption of impure B_1 concentrates, one must first, therefore, give careful consideration to the absorption of inactive purines and pyrimidines which may be present. This will probably limit the usefulness of the spectrographic method for the assay of such concentrates if vitamin B_1 itself is, as we believe, characterized by a band at 2600 Å.

The extinction coefficient (gms. per L., cms. cell thickness) of H_1 at 2600 Å. is 30.6; that of the Cerecedo preparation is 52. That of uracil (a pyrimidine) at this wave-length is 83.9. Thus the extinction of neither of these concentrates is too high to be accounted for by pyrimidine impurities of the uracil type.

COMPARISON OF THE ABSORPTION AND ACTIVITY OF THE MORE ACTIVE CONCENTRATES

Because of the absorption of inactive contaminants, cited above, one must first eliminate the less active (and therefore less pure) preparations before attempting to correlate spectra and biological activity. This has been done in preparing Table II, the H_1 and Cerecedo preparations having been omitted and the remaining concentrates (with the exception of Guha and H_1, for which quantitative values were lacking) listed in the order of their potencies. The curves of these concentrates are shown in Fig. 3.

At no wave-length does the extinction exactly follow the order of biological potency for all five concentrates. Near 2600 Å., however, the agreement is very much better than at the short- or long-wave limits of the table.

At 2250 Å., for example, Seidell 30:182 has roughly 1½ times the extinction of Seidell 30:85, yet the latter is five times

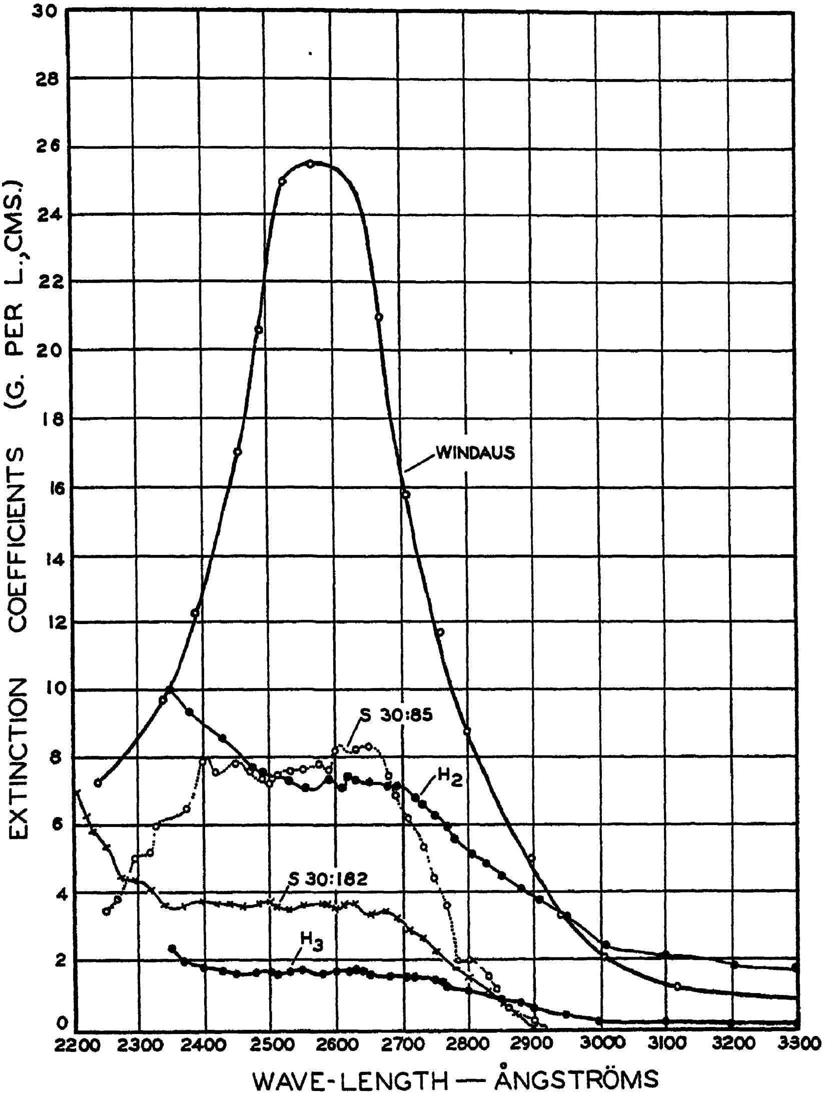
TABLE II

The More Active Concentrates.

Concentrate	Relative Order of Potency	Method of Preparation	Biological Assay		Extinction Coefficients (1 gm. per l., 1 cm.)								Relative Values, Highest =100	
			This Lab.	Other Labs.	λ =2250	λ =2300	λ =2400	λ =2500	λ =2600	λ =2700	λ =2800	λ =2900	Potency	Ext. at 2600
Windaus	1	See (5)	—	0.012	Exterpolated 7.0	Exterpolated 8.0	13.0	23.0	25.0	16.6	9.0	5.0	100	100
Heyroth (H_2)	2	Modified Method of Jansen and Donath (3)	av. = 0.045 0.04—0.05 mg.	—	Exterpolated 12.5	Exterpolated 11.0	9.0	7.5	7.2	7.0	5.3	3.8	26.7	29.8
Seidell 30:85	3	See (7)	—	0.05 mg.[1]	Exterpolated 3.5	Exterpolated 5.0	7.5	7.3	8.2	6.5	2.0	0.3	24.0	32.8
Heyroth (H_3)	4	Modified Method of Jansen and Donath (3)	av. = 0.18 0.16—0.20 mg.	—	Exterpolated 5.0	Exterpolated 3.3	1.8	1.7	1.75	1.6	1.1	0.65	6.7	7.0
Seidell 30:182	5	See (7)	—	0.25 mg.[2]	5.5	4.3	3.8	3.7	3.6	3.1	1.7	0	4.8	14.4

[1] Determined by Dr. M. I. Smith (personal communication from Dr. Seidell).

[2] Determined by Dr. M. I. Smith (personal communication).



Concentrations employed in taking the spectra: Seidell 30:85, 75 mg. per L.; H_2 and Seidell 30:182, 100 mg. per L.; H_3, 400 mg. per L.; all 2 cm. cells.

Fig. 3—Spectra of Concentrates Listed in Table II.

as potent. H_2 has a greater extinction than the Windaus preparation, yet, if the biological value assigned the Windaus preparation is correct, its potency is only about one-fourth as great. At 2900 A., H_2 has about 13 times the extinction of Seidell 30:85, the potencies being about equal, and H_1 has twice the extinction of Seidell 30:85 but is only one-fourth as potent. At these wave-lengths, the ratios of the extinction coefficients and the biological potencies deviate from each other by factors as great as 13 to 1 in one instance and 8 to 1 in several instances.

At 2600 Å., however, the relative values of extinctions and potencies agree closely. The deviation between the values based on Windaus = 100 for each (see the last two columns of Table II) is only as 27:30 for H_2, and 6.7:7.0 for H_1. In the case of the Seidell preparations, the agreement is somewhat less close, being 24:33, for Seidell 30:85, and 5:14 for Seidell 30:182. In all cases where there is even this degree of deviation, the extinction values are higher than the biological activity values. This would follow were the preparations still contaminated with inactive pyrimidines. The most serious disagreement is found in the preparation of least activity, which might be expected to contain absorbing impurities to a greater extent than the other concentrates.

When one realizes that the potencies of these preparations were measured in three different laboratories, using different methods and different animals, and that there is a likelihood that inactive pyrimidines may have been present in at least some of them, it is perhaps surprising that the degree of correlation shown by Table II could have been obtained. These data, and the fact that the curves of all of the concentrates exhibit at least an indication of an absorption maximum at 2600 Å., seem to indicate that the antineuritic factor may be characterized by a band at 2600 A. This supports in some measure the contention of Windaus that he has obtained pure vitamin B_1, in view of the marked absorption of his preparation at 2600 Å.

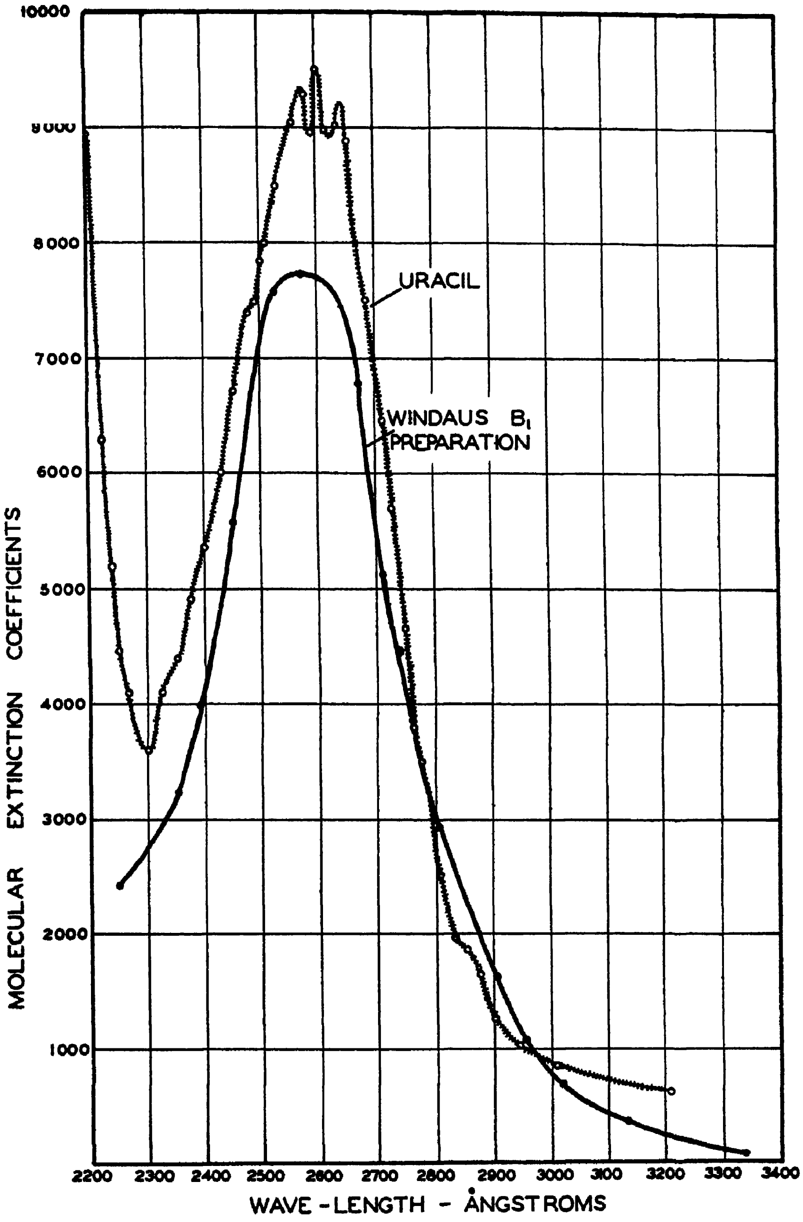


Fig. 4—Comparison of the Absorption of the Windaus Preparation with that of a Typical Pyrimidine (Uracil).

DEDUCTIONS FROM ABSORPTION SPECTRA AS TO THE CHEMICAL STRUCTURE OF VITAMIN B_1

Windaus (5) presented his absorption data in the form of a curve giving the absorption coefficients of a 0.02% solution of the hydrochloride in alcohol. By the term "absorption coefficient" is generally understood $\frac{1}{cd} \log_e \frac{I_0}{I_\lambda}$. Our ordinates are in terms of "extinction coefficients", that is, $\frac{1}{cd} \log_{10} \frac{I_0}{I_\lambda}$. We have, therefore, multiplied the Windaus ordinates by 0.434 to change them to the basis of Briggs' logarithms ($\log_{10}$), as well as by 10 to convert from mm. thickness to cm. thickness, and by 5 to convert from the 0.02% concentration to one of 1 gm. per liter. Finally, we have multiplied them by 325, the molecular weight of the dihydrochloride indicated by the analysis (neglecting the possibility of polymerization) presented by Windaus. The values so obtained are plotted in Fig. 4, in which the molecular extinction coefficients of a pyrimidine (uracil), are also presented for comparison. Not only is a striking similarity in the forms of the curves apparent, but the probable Windaus molecular extinction coefficient of 8225 at 2600 Å. is very close to the value 9500 of the pyrimidine (2) at the same wave-length.[3]

This close agreement appears to indicate the presence of a pyrimidine or closely related ring in the Windaus preparation. The magnitude of the Windaus absorption in comparison with that of certain pyrimidines indicates that the absorbing material constitutes at least a large fraction of the preparation. If this absorbing material is not the true vitamin B_1, but is only contaminated by the latter, then it would necessarily follow that the potency of B_1 itself is many times as great as that determined by Windaus for his preparation. It is inadvisable to attempt a more quantitative consideration of this possibility, as it is difficult to estimate the influence of such factors as the use of alcohol as the solvent in the case of the Windaus curve, and the presence of sulphur, and of other substituents implied by the empirical formula.

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[3] If the molecular weight, 357, corresponding to the formula of van Veen (8) is employed, the molecular extinction of the vitamin crystals becomes 8925.

Some speculation as to the nature of the absorbing compound may perhaps be justified. That it contains a pyrimidine ring seems likely. That other types of nitrogenous heterocyclic compounds, the absorptions of which have not yet been studied, may have a similar type of absorption cannot, however, be excluded. The absorption of the few hydantoin and glyoxalone compounds studied does not in general resemble that of the pyrimidines, although they are closely related to them chemically and biologically. There is, however, one exception. Holiday (12) has found that the absorption of ergothioneine, $C_9H_{15}N_3O_2S$, a betaine of thiolhistidine which has not been noted as occurring in yeast, has a maximum at 2570 Å. with a molecular extinction coefficient of the order of that of the purines. In the present rather undeveloped state of knowledge of the absorption of the nitrogenous ring compounds, the two types of compounds most likely to give absorption spectra similar to that of the Windaus preparation appear to be pyrimidine-ring containing compounds, or compounds closely related to ergothioneine.

Both Windaus (5) and Seidell (7) have found sulphur in their concentrates, an observation which is especially stimulating in view of the work of Reimann and Hammett (13) on the effect of certain organic sulphur compounds on cellular proliferation, and of the finding by Cerecedo (14) of a disturbed sulphur metabolism in the dog after feeding certain pyrimidines. The empirical formulas assigned to the crystalline B_1 preparations by Windaus ($C_{12}H_{17}N_3O_2S$) and by van Veen ($C_{12}H_{20}N_1O_2S$) indicate that, if the absorbing substance is a pyrimidine, there is, in addition to the sulphur, a considerable number of carbon and hydrogen atoms to be accounted for. These might be present as substituents at several positions in the ring, or, with less likelihood of interference with the absorption, they might be present in a nucleoside. A sulphur-containing nucleoside, adenine thiomethylpentoside, it may be recalled, was isolated from yeast by Suzuki, Odake and Mori (15). Against the possibility that the absorbing compound may be a nucleoside is the fact that the empirical formulas which have been proposed for it have less oxygen than would be demanded by the naturally occurring nucleosides at present known. The formula for ergothioneine, however, differs from that proposed by Windaus for his B_1 preparation only in lacking three carbon and two hydrogen atoms, and from the van Veen formula by lacking one nitrogen, three carbon, and five hydrogen atoms.

THE ABSORPTION OF OTHER FACTORS OF THE B COMPLEX

The correlation of vitamin B_1 activity with ultra-violet absorption is complicated by the existence of several B factors with distinct physiological functions. As to the presence or absence of these factors in the concentrates studied, we have but little information. We ourselves made no tests for B_2. It is, however, recognized (16) that preparations made by the method employed by us for the preparation of H_4 lack this factor. No information is available as to the presence of B_4 in the Windaus, Seidell, or Cerecedo concentrates. In the case of our H_1, H_2, and H_3 preparations, we felt (3) that the biological assay methods employed had not been sufficiently developed to permit a discrimination between B_1 and B_4. Our preparation, H_4, however, has not only produced prompt cures of the convulsive symptoms and paralyses attributed by Kinnersley, Peters, and Reader (17) to the factor B_1, but has also caused an immediate and pronounced increase in weight, and has usually, but not uniformly, cleared up the residual symptoms (with the exception of swollen red paws which we have never encountered) attributed by these workers to a deficiency in B_4. This tends to confirm the statement of Miss Reader (18) that preparations made by this method mitigate the symptoms attributed both to the B_1 and B_4 factors.

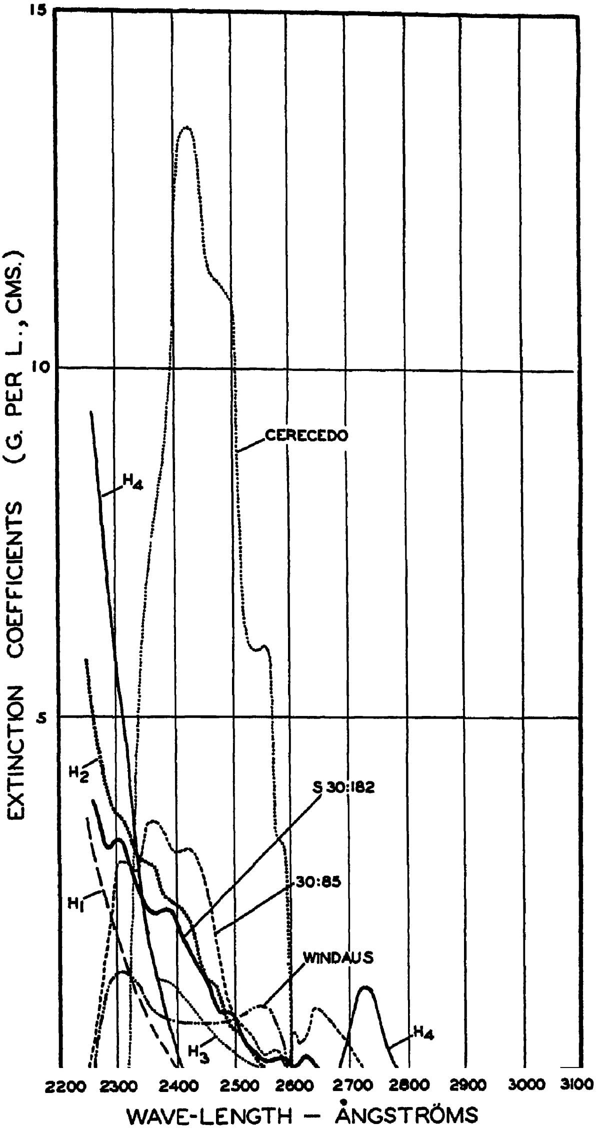

Fig. 5—Difference Curves Showing the Residual Absorption of Various Concentrates after Subtracting the Absorption of Thymus Nucleic Acid.

In considering the possible influence of factors other than B_1 upon the absorption of these concentrates, it may be noted that the curves of Fig. 1 fall into two classes. Some of these curves resemble those of pyrimidines or nucleic acids; the others rise rapidly in the shorter-wave region, indicating the presence of compounds having chiefly "end" (or shorter-wave) absorption. This second type of curve is, perhaps, a composite of absorption of the pyrimidine type with that of substances having "end" absorption, and but little absorption in the region near 2600 Å. Certain nitrogenous ring compounds related to the pyrimidines, such as isodialuric acid, alloxan, and diisobarbituric acid (unpublished observations) absorb after this fashion.

To render more evident the presence of absorption of this type in the concentrates, we have subtracted from each of the curves the absorption of thymus nucleic acid (as typical of the pyrimidine type of absorption) plotted on such a scale that the nucleic acid absorption and the absorption of the concentrate in question were the same at 2600 Å. The resulting difference curves, reproduced in Fig. 5, show in the case of the H_2, H_3, and Seidell 30:182 preparations, absorption rising rapidly from about 2600 Å. toward the shorter wave-lengths. The difference curve of the Seidell 30:85 concentrate has a pronounced maximum at 2360 Å., and possibly lesser ones at 2310 and 2420 Å. That of the Cerecedo preparation shows the presence in large quantities of a substance or substances having pronounced absorption with a maximum at about 2425. That the difference curve of the Windaus preparation shows comparatively little absorption, notwithstanding the marked absorption of the concentrate itself, indicates that this preparation is relatively free from materials having absorption other than that of the pyrimidine type. The H_1 and H_4 difference curves rise rapidly from about 2400 Å., indicating the presence of substances with end absorption, but not of the same type as that found in the Seidell 30:182, H_2, and H_3 concentrates.

Any attempt to correlate biological activity and end absorption of either type—that rising from 2600 Å. or from 2400 Å.—would at present be premature. The residual absorption in the

2300-2500 Å. range, as found in the Cerecedo and Seidell 30:85 concentrates, is probably not attributable to the factor B_1, since preparation H_4, which apparently contains this factor, does not exhibit this type of residual absorption. Should this type of absorption represent any biological factors, it could probably be only B_2 or the lesser studied B_3 (19), B_5 (20), or Chick's "Y" factor (21).

CONCLUSIONS

1. The presence of biologically inactive purines and pyrimidines complicates attempts to correlate the ultra-violet absorption of vitamin B_1-containing concentrates with their antineuritic potencies.

2. In selected concentrates, there is a sufficiently close correlation between the B_1 activity and the absorption at 2600 Å. to indicate that this factor is characterized by an absorption maximum at 2600 Å.

3. The similarities of the absorption spectra to those of pyrimidine-ring-containing compounds and compounds of the type of ergothioneine indicate that vitamin B_1 may be a compound of one of these two types.

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THE FORMATION OF VITAMIN A IN CORN SPROUTS BY LIGHT, AND THE TRANSFER OF THE VITAMIN FROM THE SPROUT TO THE GRAIN

By G. PARK GOODE

SUMMARY

White field corn was germinated in the dark, and the sprouts allowed to grow to a length of about two inches. Part of the sprouts were then irradiated with the light from a Mazda lamp in such a way as to protect the grain from the light. After irradiation, the grain was cut free from the sprouts and fed to rats on a vitamin A-free diet. The grain from irradiated sprouts had greater potency in relieving vitamin A-deficiency symptoms than that from unirradiated sprouts. It is concluded that the irradiation increased the vitamin A content of the sprouts, and that the vitamin was transferred in part from the sprouts to the grain during the irradiation process.

During the course of some investigations in progress in this laboratory on the influence of light on the formation of vitamin A in plants, a marked increase was noted in the quantity of the vitamin in corn sprouts that were irradiated after being cut from the grain as compared with that found in sprouts that were irradiated while still growing normally.

After consideration of the several factors that could effect this result, the most logical explanation was thought to be that any vitamin formed in the shoot during irradiation may have been transferred to the grain through the agency of the usual growing processes. The interruption of such growing processes by severing the shoot from the grain would thus result in the vitamin remaining in the shoot after formation by irradiation.

Coward (1) has reported work showing that such a transfer of the vitamin takes place in carrots. If light is the principal factor in the formation of vitamin A, some mechanism of transfer must be responsible for the relatively high content of the vitamin in such vegetables as carrots and sweet potatoes which are in almost complete darkness throughout their growing period, only the leafy portion of the plant being exposed to light.

The following experiments were undertaken to determine if vitamin A is transferred from the sprout to the grain when the sprout is irradiated.

White field seed corn was germinated at about 37° C. on damp white sand. It was protected from light throughout the germination and growing periods. When the sprouts were about two inches long the grain for one group of rats was prepared by removing the sprouts, placing the grain on damp sand, and covering it with black paper. For the other group of animals, the grain with the sprouts still attached was placed on damp sand and a piece of black paper with holes punched in it placed over the grain, allowing the sprouts to extend through the holes. In this way the sprouts only were irradiated, the grain being protected by the black paper.

Both lots of grain, protected by the black paper, were placed in the same tray during irradiation insuring their preparation under identical conditions of temperature, humidity and atmosphere.

The sprouts so prepared were irradiated for sixteen hours at a distance of 6 inches from a 50 watt, inside frosted, Mazda C lamp, in an aluminum-painted desk lamp reflector.

After irradiation, the sprouts were cut from the grain, care always being taken to remove the entire sprout. The rats were each fed one gram of the moist grain daily. The dosage level of one gram was selected for this preliminary experiment in order to give some indication of the proper range of dosages for later, more refined experiments. It does not represent the minimal dose desirable for detecting small differences in vitamin potency.

Two groups of rats, one of four animals and one of five, were fed the vitamin A deficient ration in use in this laboratory (2).

At the beginning of the experiment, the rats weighed 35 to 45 grams. After about 21 days, the weight curves began to show the characteristics of vitamin A deficiency and feeding of the grain was begun.

Shortly after the grain feeding was started, the vitamin B content of the dried yeast component of the diet was found to be exceedingly low. For this reason the slackening of growth in the animals could not be attributed entirely to lack of the vitamin A. By the time this abnormal deficiency was detected and corrected, the weight curves had become somewhat erratic, obscuring any critical analysis. However, as many investigators have indicated that weight curves are not of major significance except at minimal dosages, the experiment was continued with the intention of relying on xerophthalmia as the criterion of vitamin A potency.

After 30 days of feeding the grain supplement, all but one rat in the group receiving the untreated grain developed severe xerophthalmia, which persisted in each case until death of the animal. The remaining rat in this group increased in weight slightly for about ten days and then declined steadily until the end of the experiment.

In the group that was fed the grain after irradiation and removal of its sprouts, all rats made good weight gains, were of excellent appearance, and exhibited no symptoms of xerophthalmia.

Despite the fact that the weight curves were not entirely acceptable as evidence, the uniform development of xerophthalmia in the one group and the total freedom from it in the other seems reasonably conclusive proof of a higher vitamin A content in the grain from the irradiated sprouts.

Since the grain for both groups of animals was germinated and the sprouts grown under exactly the same conditions, and since the grain was kept under identical conditions during the irradiation of the sprouts, the increase in vitamin A content in the irradiated lot must be due to the transfer of the vitamin from the sprout to the grain through some mechanism of the growing process.

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VITAMIN D—A REVIEW

PART I—ULTRA-VIOLET ABSORPTION SPECTRA IN RELATION TO VITAMIN D—(Continued)

BY JOHN R. LOOFBOUROW

SECTION D—CHANGES IN THE ABSORPTION SPECTRUM OF ERGSOTEROL ON IRRADIATION

SUMMARY

Pohl (12), who was apparently the first to publish such studies, concluded from difference curves that the vitamin D in irradiated ergosterol is characterized by an absorption band at 2450 Å. Morton, Heilbron, and Kamm (91) came to similar conclusions independently. Rosenheim and Webster (39) criticized Morton, Heilbron, and Kamm's conclusions, but the latter replied (92) with confirmatory evidence. Van Stolk, Dureuil, and Heudebert (93) found a band at 2500 Å. and lesser bands at 2600 and 2405 Å. in irradiated solutions. Smakula (94) criticized earlier work because of the possible effects of oxidation, etc. He obtained difference curves from solutions irradiated with magnesium spark light which indicated vitamin D to have bands at 2620 and 2930 Å. Bills, Honeywell, and Cox (95) found that the intensity of the 2450 Å. band did not parallel the biologic potency of irradiated solutions. They suggested that this band might be due to isoergosterol. Reerink and van Wijk (96) independently made a similar suggestion. Kon (97) warned against the premature acceptance of the 2450 Å. band as characteristic of the vitamin. A number of workers, as Morrison and Clark (98), Fabre and Simonnet (99, 100, 101, 102), and Castille and Ruppol (104) published results, about this time, indicating the presence of the short-wave band in irradiated solutions. Fabre and Simonnet (100) and Delaplace and Rebière (103) concluded, in agreement with Bills, Honeywell, and Cox (95) that maximum short-wave absorption develops some time after maximum potency is reached. Webster and Bourdillon (105, 106, 107) found marked absorption at 2800 Å. in samples of ergosterol irradiated for short periods. They attributed this absorption to a product "A" which they believed to be identical with vitamin D. This product was presumed to be decomposed by further irradiation into "B", an intermediate product with a band at 2450 Å., and "C", a final product with negligible absorption. By the use of filtered ultra-violet rays, Reerink and van Wijk (108) concluded that the substance with marked absorption at 2800 Å., which they termed "S", was an inactive product of the short waves, and that the long-wave product, which they termed "L" and found to be characterized by a band at 2600 Å., was vitamin D. Everse and van Niekerk (109) found the biological potency of Reerink and van Wijk's samples to parallel closely their content of "L". Bourdillon, Fischmann, and Jenkins (110) reaffirmed the position of the English workers regarding the relation of 2800 Å. absorption to the vitamin, but shortly afterward, Bourdillon, Jenkins, and Webster (111) announced that the supposed relationship had been fortuitous and that Reerink and van Wijk were probably correct. Several later papers, notably those of Holtz (113), Windaus (114), Reiter (115), and Reerink and van Wijk (116, 117) indicated that the 2600 Å. band in irradiated ergosterol solutions could probably be attributed to the vitamin. Marshall and Knudson (118) suggested that vitamin D absorbs in the same region as ergosterol, and Cox and Bills (119) again called attention to the similarity of the 2450 Å. band to that of isoergosterol. The evidence at the close of this period thus favored the view that the vitamin was characterized by a band at 2650 Å., and that the bands at 2450 and 2800 Å. was due to inactive irradiation products.

How ergosterol came to be accepted as the precursor of vitamin D has already been outlined.[1] With the adoption of this point of view, it was natural for investigators to turn next to a study of the absorption changes induced in ergosterol by irradiation with ultra-violet light. In this way, they hoped to determine the absorption of the vitamin and to deduce therefrom its chemical structure. Other workers (as for example, Windaus and his associates) concerned themselves primarily with attempts to prepare the vitamin in a pure state. We shall come to that matter shortly—for the present we shall confine ourselves as closely as possible to a discussion of those papers dealing with the absorption of the crude products arising from the irradiation of ergosterol.

During the course of these investigations, three main hypotheses regarding the absorption of the vitamin were successively put forward. The first characterized it by a prominent band near 2450 Å. This was the result, as we shall see, of the examination of over-irradiated ergosterol solutions. The second placed the band near 2800 A. and gave it greater extinction than that of ergosterol. This observation apparently arose from the presence of inactive irradiation products, formed simultaneously with the vitamin. The third fixed the band near

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[1] Pages 101-156, vol. 3, this journal.

2650 Å., a point of view not inconsistent with the most recent investigations of crystalline D preparations. This hypothesis was the result of careful investigations of the effects of filtered radiations on the photo-activation process.

THE 2450 A ABSORPTION BAND

The pioneer in these absorption studies was, so far as we have been able to learn, R. Pohl (12; 1927). His paper has already been discussed,[1] and his curves reproduced (Figure 6). He subtracted the curve of ergosterol from that of irradiated ergosterol and obtained a curve with a pronounced maximum at 2450 A., which he attributed to vitamin D.

Independently, Morton, Heilbron, and Kamm (91; 1927) came to similar conclusions.

Using a 0.2 g. per liter solution of ergosterol in "optically pure" alcohol, they carried out two series of irradiations with a quartz mercury arc, removing samples every 15 minutes and determining their absorption spectra (A6). In this way, they obtained two "reasonably concordant" sets of curves, one of which is presented in their paper and reproduced here (Figure 42).

RELATION OF THE 2450 A BAND TO VITAMIN D EMPHASIZED

How they, like Pohl, concluded from these curves that vitamin D is characterized by a maximum near 2450 Å. is best told in their own words:

"The curves show that the reaction:

$$\text{ergosterol} \xrightarrow{\text{u. v. light}} \text{vitamin D}$$

is accompanied by the disappearance of selective absorption in the region 260-300 $\mu\mu$ and by the appearance of a new selective absorption in the region 230-260 $\mu\mu$ with a maximum at 247 $\mu\mu$. This new band will itself disappear with further irradiation, a fact which is not inconsistent with the view that it is due to vitamin D, since it is known that 'excessive' irradiation of cod-liver oil induces a loss of vitamin potency."

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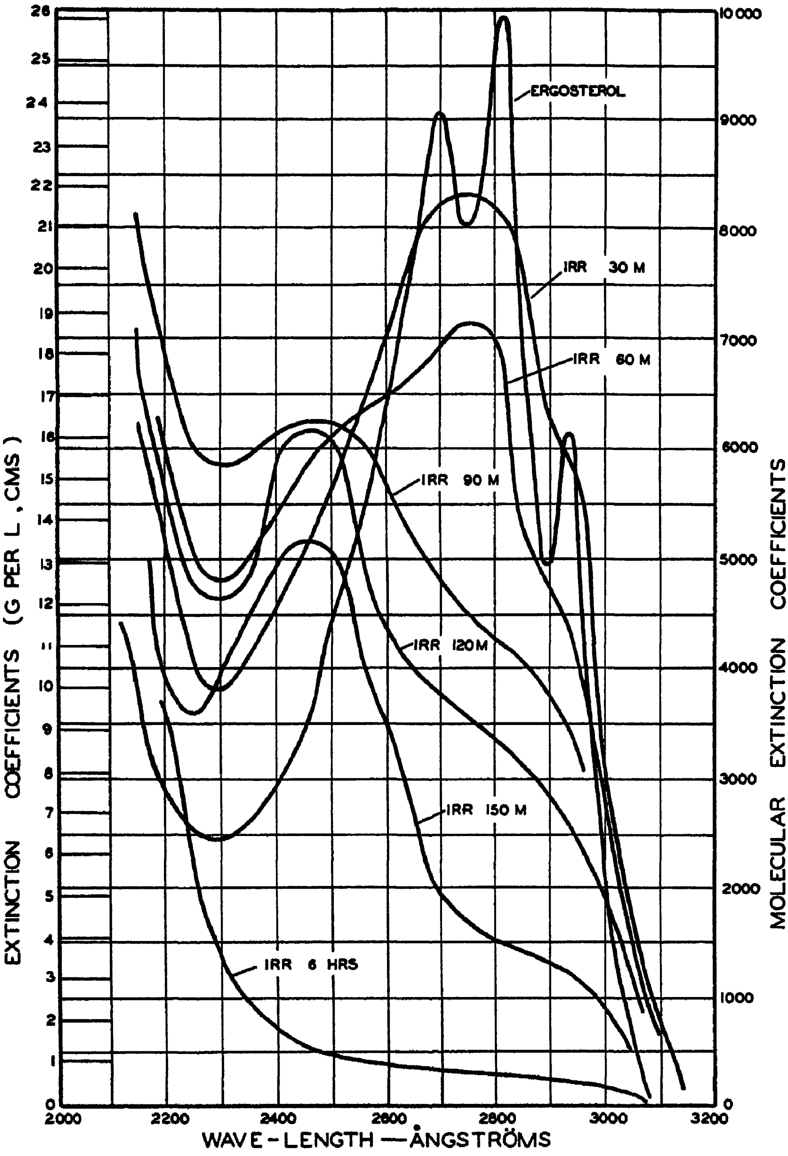
[1] Pages 124-126, vol. 3, this journal.



0.2 g. per L solutions in alcohol (CC).

Fig. 42—Absorption of Ergosterol Irradiated with Full Ultra-Violet for Various Times [Morton, Heilbron, and Kamm (91; 1927)].

Incidental biological experiments seemed to support their deductions.[1]

They concluded that to produce vitamin D without destroying it, one should filter out the rays in the region of the 2470 Å. band (namely, all radiations shorter than about 2700 Å.).[2]

ROSENHEIM AND WEBSTER QUESTION THE IDENTITY OF THE 2450 Å. BAND WITH VITAMIN D

These observations did not go unchallenged for long. Rosenheim and Webster (39; 1927) published a severe criticism of them, aimed mainly, however, at the conclusions regarding filtering (discussion of which will be reserved until later).

Heilbron, Kamm, and Morton (92; 1927) in answering Rosenheim and Webster's criticisms, said that Mr. A. E. Gillam had found the 2470 Å. band to remain evident for a long time in solutions of ergosterol irradiated under conditions similar to those under which Rosenheim and Webster (39) found the biological activity to persist for long periods.

PRESENCE OF THE 2450 Å BAND IN IRRADIATED ERGOSTEROL CONFIRMED

Van Stolk, Dureuil, and Heudebert (93; 1928), after discussing some experiments with filtered radiations,[3] described studies of the absorption changes produced by full ultraviolet in alcoholic solutions of ergosterol under nitrogen. Under these conditions, pronounced absorption in the shortwave region, with a maximum at about 2500 Å., was found to be produced after the destruction of the 2700, 2815, and 2932 Å. ergosterol bands (Figure 43). Their curves (Ae5) thus confirmed qualitatively the absorption changes observed by Morton, Heilbron, and Kamm (91), but instead of a single band near 2470 Å. in the irradiation product, they found lesser bands also at 2600 and 2405 Å. After four hours' irradiation, the short-wave bands had not diminished, and even after six hours they were present, though somewhat decreased in height.

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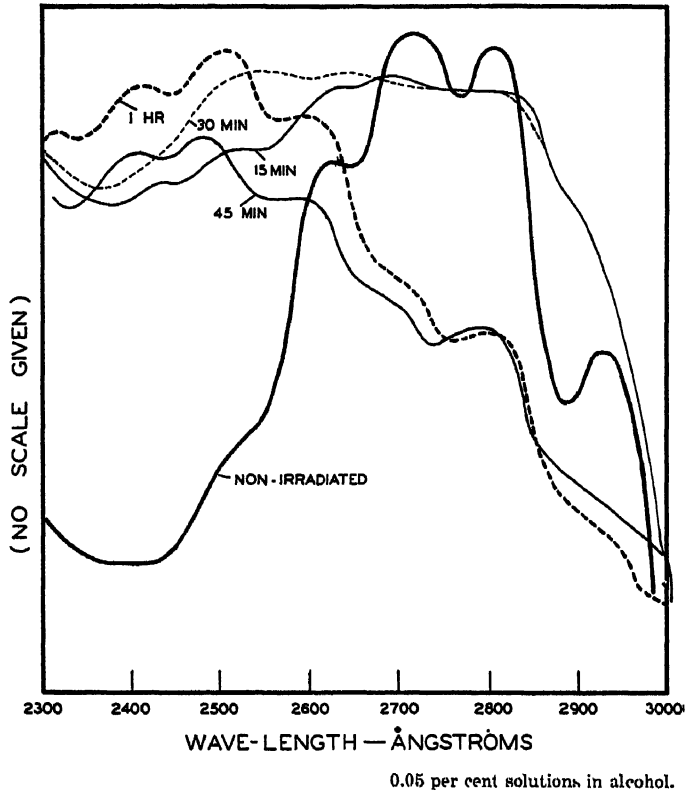
[1] A footnote stated that Messrs. H. Jephcott and A. L. Bacharach performed animal experiments in which the product with the most pronounced 2470 Å. band was found to be highly potent, while the product which had been irradiated until this band disappeared was inactive at the same dosage. We now know, of course, that such results could readily have been obtained even if the product with pronounced absorption at 2470 Å. contained mainly over-irradiation products, provided only that over-irradiation had not been carried so far as to destroy all measurable vitamin potency.

[2] The discussion of this matter belongs in a later section.

[3] These will be considered in the proper place.

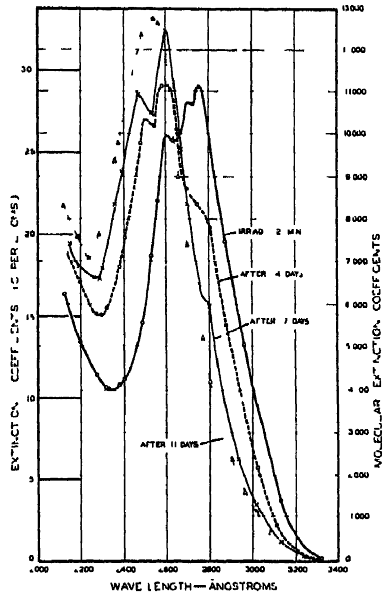


0.05 per cent solutions in alcohol.

Fig. 43—Absorption of Ergosterol Irradiated Under Nitrogen with Full Ultra-Violet [Van Stolk, Dureuil, and Heudebert (93; 1928)].

SMAKULA'S STUDIES OF THE EFFECTS OF OXYGEN

Early in 1928, a German investigator, Alexander Smakula (94), questioned the conclusions of Morton, Heilbron, and Kamm that the 2450 Å. band represented vitamin D (91, 92), because they did not state whether absorption measurements had been made in all cases immediately after irradiation, and whether adequate precautions had been taken to exclude oxygen. He considered both of these factors important, and believed that they might have led the English workers to erroneous conclusions.

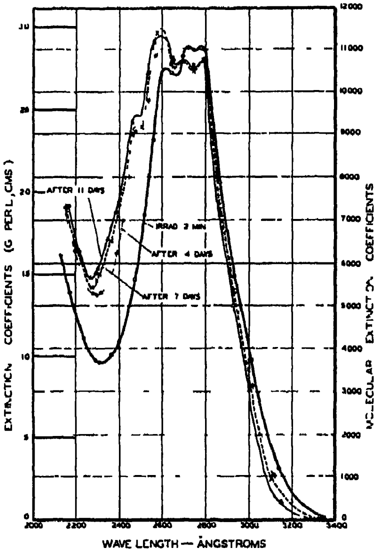


0.1 g. per L. solutions in ether (AAaaI).

Fig. 44—Changes in the Absorption of Irradiated Ergosterol Solutions After Standing in the Presence of Air [Smakula (94; 1928)].

To determine the influence of these factors, he observed the spectra of irradiated solutions allowed to stand in the presence of oxygen and found that they changed materially (Figure 44). The absorption changed somewhat, but not as markedly, if the solutions were kept in oxygen-free containers (Figure 45). Smakula was inclined to attribute this to traces of oxygen left in the ether or bound chemically to the crystalline ergosterol, though he suggested also the possibility that the first reaction products might have reacted with the ergosterol upon standing.

In order to eliminate oxidation changes from the photodynamic picture, he carried out a series of measurements on magnesium-spark-irradiated ergosterol solutions in which air was eliminated from the cells and the absorption was measured in each case promptly after irradiation.



0.4 g. per L. solutions in ether (AAaal).

Fig. 45—Changes in the Absorption of Irradiated Ergosterol Solutions After Standing in Full Cuvettes [Smakula (94; 1928)].

USE OF DIFFERENCE CURVES TO OBTAIN THE ABSORPTION OF THE VITAMIN

From the curves of the irradiated materials (Figure 46) he subtracted the absorption of unchanged ergosterol, after multiplying the latter in each case by a factor which he presumed to represent approximately the amount of unchanged ergosterol remaining in solution. The multiplying factor used for the 2½-second irradiated sample[1] was 0.9, and it was assumed that the ergosterol was always reduced by about 5 per cent by doubling the time of exposure.

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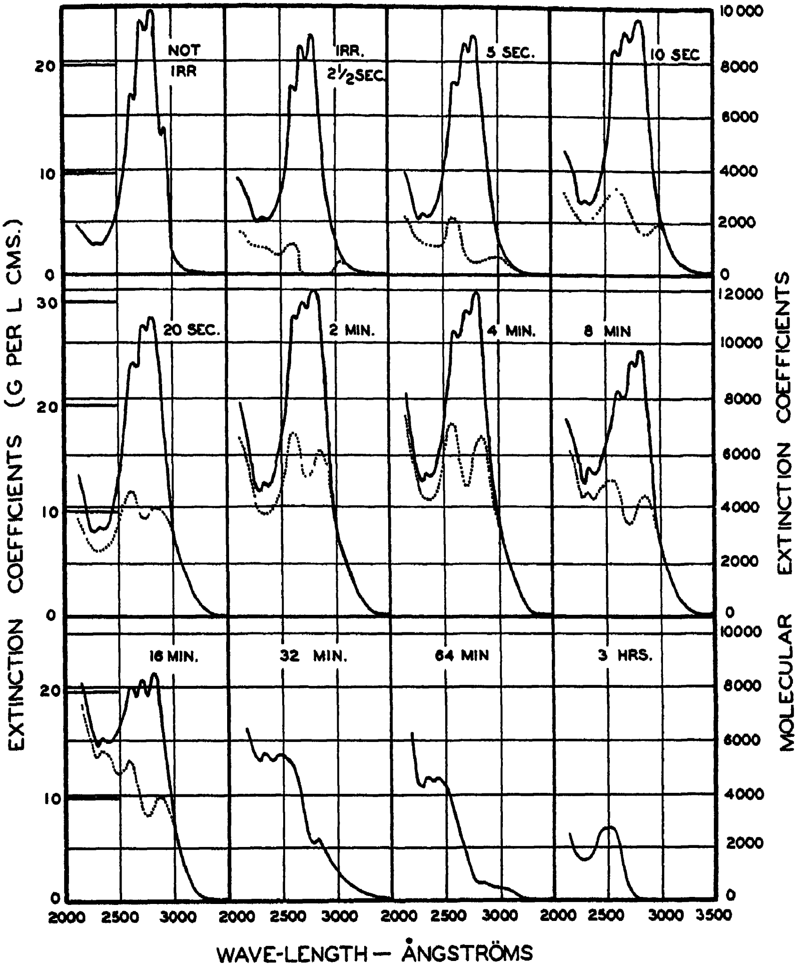
[1] It is stated that this sample was found by Dr. Windaus to be biologically active.



0.4 g. per L. solutions in ether (AAaaI).

Fig. 46 --Absorption of Ergosterol Irradiated in Full Cuvettes with Magnesium Spark Radiations [Smakula (94; 1928)].

The dotted curves show the residual absorption after subtracting the absorption of the unchanged ergosterol presumed to be present in each of the solutions (see text). Smakula's published curves of the irradiated solutions showed the experimental points, but the reproduction was so small that it was impracticable to show the points here, hence they were omitted.

While it was the possible effects of oxygen and standing that inspired Smakula's more refined study of the problem, the value of his contribution lay mainly in that he was the first to examine solutions irradiated for periods sufficiently short to insure that they contained but small proportions of over-irradiation products.

PRESENCE OF 2620 Å. AND 2930 Å BANDS IN EARLY IRRADIATION PRODUCTS

His difference curves for the early stages of irradiation (Figure 46) were quite at variance with the conclusions of Pohl (12) and Heilbron, Kamm, and Morton (91, 92) that the first irradiation product (presumably vitamin D) was characterized by a band with a maximum near 2450 Å. In Smakula's words: "In the photochemical activation of ergosterol there is formed first a substance which, of the four principal absorption bands of ergosterol, still shows only the two at 293 and 262 mμ."

This conclusion led him to propose that the first irradiation product "is an ergosterol in which two of the four principal bands of ergosterol have been removed due to the removal of some internal molecular linkage".

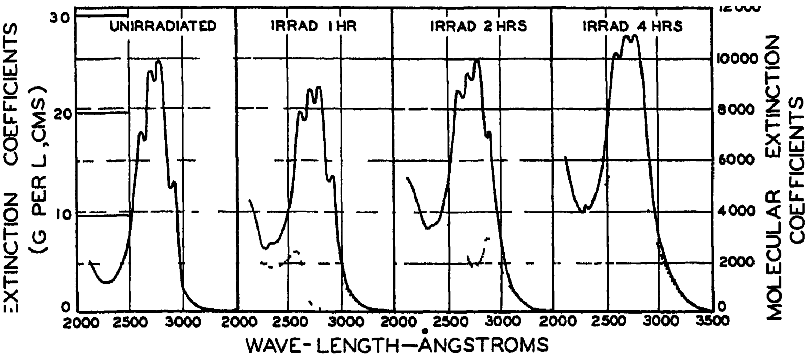
EFFECTS OF MONOCHROMATIC LIGHT

Smakula also studied the effects of monochromatic light of wave-length 2800 Å., obtained with a monochromator and a magnesium spark source, on the absorption spectrum of ergosterol (Figure 47). Because of the greatly decreased intensity of the light, four-hour exposures to the monochromatic light (the longest ones employed), were only equivalent to about two-minute exposures to full Mg spark light. When allowance was made for this difference in intensity (by comparing the 1-hour, 2-hour, and 4-hour monochromatic curves with the 10-second, 20-second, and 2-minute full Mg spark curves, respectively) the effects of full Mg spark light and 2800 Å. monochromatic light appeared to Smakula to be about the same for these early periods of irradiation. One would hardly have expected a great difference, however, since Mg spark light itself is richest in radiations near 2800 Å.

A point that Smakula did not mention is that the 2620 Å. maximum seemed to be somewhat more prominent in comparison with the 2930 A. maximum in the curves of the monochromatically irradiated materials. The importance of this difference, if it may really be accepted as true, will appear later.

APPEARANCE OF THE 2450 A BAND AFTER PROLONGED IRRADIATION

On prolonged irradiation, Smakula's difference curves for full Mg spark light showed a degradation of the 2930 and 2620 A. bands and the appearance of the previously observed band near 2450 Å. (Figure 46). The results indicated that those who believed the 2450 Å. band to represent the vitamin had been dealing with over-irradiation products, and that the true vitamin bands were more probably near 2600 and 2900 A. Smakula suggested only these conservative conclusions, however: "Now since this first absorption product is antirachitically active, one is no longer entitled to disregard, as unimportant in physiological experiments, a residual absorption remaining in the region of 280 mμ. Consequently, we have irradiated an ergosterol preparation long enough to remove any absorption in the region of 280 mμ, and therewith the first reaction product (Figure 46). The physiological investigation of this preparation is necessary in order to come



0.2 g per L solutions in ether (AAaaI).

Fig. 47 Absorption of Ergosterol Irradiated in Full Cuvettes with 2800 Å. Monochromatic Radiations [Smakula (94; 1928)]. (See note under Fig. 46).

to a decision. If it is no longer active, the reaction product with a maximum at 247 mμ can no longer be claimed to be the vitamin." Thus, as far as Smakula was concerned, the question of the non-identity of the band near 2450 Å. with the vitamin was still left open, awaiting biological confirmation.

PARALLEL BIOLOGIC AND SPECTROGRAPHIC TESTS

This confirmation soon appeared in a paper reporting a carefully-controlled series of parallel biologic and spectrographic tests of irradiated ergosterol, published in the U. S. A. by Bills, Honeywell, and Cox (95; 1928). Cognizant of the work of Pohl (12) and Morton, Heilbron, and Kamm (91, 92) though apparently not of that of Smakula (94), they believed a correlation of the biological assay with spectrographic tests to be advisable in order to establish what, if any, ultra-violet absorption might truly be attributed to vitamin D.

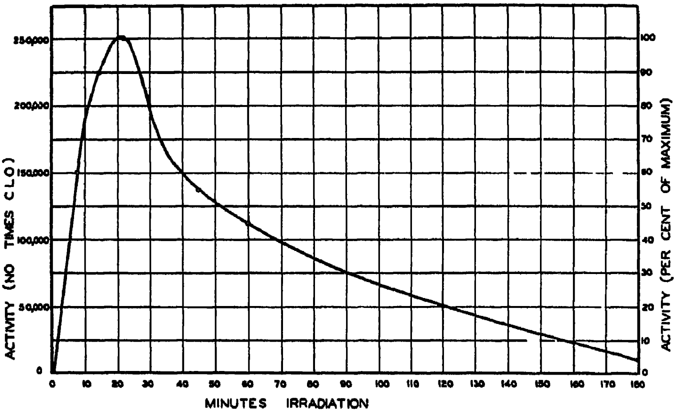
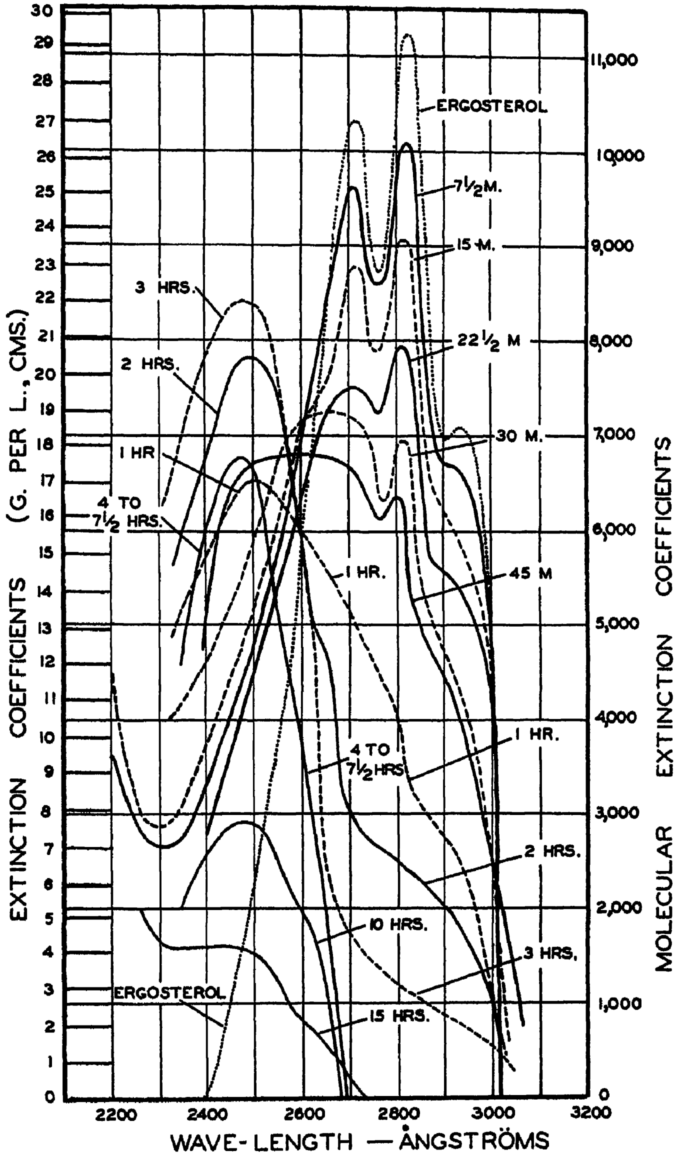


Fig. 48—Activation Curve of Ergosterol Irradiated with Full Ultra-Violet, as Determined by Biologic Experiments with Rats [Bills, Honeywell, and Cox (95; 1928)].

They used 2 cm. quartz cells, directly in contact with the window of a water-cooled Kromayer lamp, filled with a 1 g. per liter solution, in "optically pure" 95 per cent alcohol, of an ergosterol prepared by themselves ($([\alpha]_D^{20} = -132°$ in $CHCl_3$). A fresh cellful of solution was used for each different time of irradiation.



0.1 g. per L. solutions in 95 per cent alcohol (CC).

Fig. 49 —Absorption of Ergosterol Irradiated with Full Ultra-Violet for Various Times [Bills, Honeywell, and Cox (95; 1928)].

The biological activity of their samples was determined by the line test, with rats. Each point on their "activation curve" (Figure 48) represented the average of tests on about twenty rats.

POTENCY AND 2450 Å BAND NOT CORRELATED

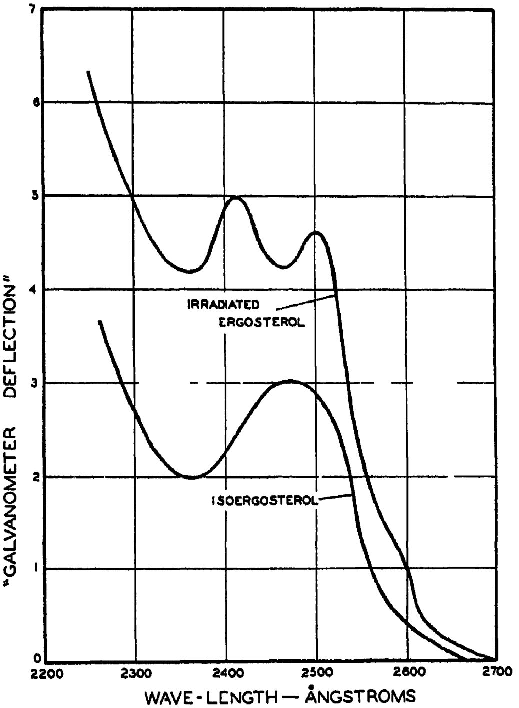
Comparison of the "activation curve" (Figure 48) with the spectrographic curves (Ab6) (Figure 49) showed that maximum potency was attained before the band near 2450 A. became prominent, and that by the time the band reached its maximum, the potency had largely been destroyed. "It is thus evident," said the authors, "that the photochemical reaction product which exhibits the absorption band at 248 mμ is not vitamin D; for its appearance coincides not with the development but with the destruction of antirachitic potency. It must be either a by-product of the activation of ergosterol, or a degradation product of the vitamin."

They did not suggest any specific bands as truly characteristic of the vitamin. They did say that "the wave-lengths which vitamin D itself absorbs, and by which it is destroyed, apparently lie within the same spectral region as the wave-lengths which activate ergosterol", but since one knows that ergosterol is activated throughout the wide region 3100-2000 Å., this was hardly more than saying that vitamin D has absorption somewhere in the ultra-violet region.

They repeated their experiments using the technique of Morton, Heilbron, and Kamm (91) in which air was admitted to the cell during the course of irradiation by the removal of samples from time to time. Under these conditions, the band near 2450 Å. developed and faded more rapidly, never reaching as great extinction, but the activation curve was much the same. "It seems obvious," they concluded, "that oxidation is involved in the destruction of the 248 mμ band, but not in the formation of vitamin D."

IS THE 2450 Å BAND DUE TO AN ISOERGOSTEROL?

Finally, they suggested that the band near 2450 A. might have been due to isoergosterol or a substance of similar molecular configuration. They observed the absorption of the isoergosterol of Reindel, Walter, and Rauch (34; 1927) and found it to have a band from about 2200 to 2700 Å., with a maximum at 2480 A., and a molecular extinction coefficient of 16,300. Prolonged irradiation caused this band to fade.



Irradiated ergosterol, 0 01 per cent solution in alcohol, isoergosterol, 0.008 per cent solution in alcohol.

Fig. 50 Comparison of the Absorption of Irradiated Ergosterol and Isoergosterol [Reerink and van Wijk (96; 1928)].

Reerink and van Wijk (96; 1928) independently concluded that the band near 2450 A. might be due to isoergosterol. At that time, they were apparently of the opinion that the band near 2450 A. was associated with vitamin D. They compared the absorption of Reindel's isoergosterol with that of a 0.01 per cent alcohol-ergosterol solution, irradiated until absorption in the 2700-3000 A. region had disappeared (Figure 50). The two curves were found to be grossly similar, but there were three small peaks in the irradiated ergosterol curve, at 2620, 2500, and 2420 Å., not found in isoergosterol. They identified the peaks at 2620 and 2500 Å. with corresponding peaks in unirradiated ergosterol,[1] but said the meaning of the 2420 Å. peak was not clear. To explain the various bands, the following hypothesis was suggested: Ergosterol has two systems of bands, associated with different parts of the molecule. The 2930, 2810 and 2700 bands are associated with a part of the molecule which on irradiation undergoes a structural change similar to that brought about chemically in the preparation of Reindel's isoergosterol from ergosterol. The 2620 and 2500 Å. bands are characteristic of a part of the molecule which does not change constitutionally upon irradiation.

OTHER PAPERS RELATED MAINLY TO THE 2450 Å BAND

The remaining papers relevant to the matter of the 2450 Å. band are not of great consequence, but should be considered briefly for the sake of completeness.

Kon (97; 1928) incidentally mentioned the band in one of his papers on the photochemistry of ergosterol,[2] saying that it was premature to accept it as characteristic of vitamin D in view of the evidence that the photochemical transformation of ergosterol, even in monochromatic light, was quite complicated.

Morrison and Clark (98; 1929) investigated the absorption changes of ultra-violet irradiated ergosterol qualitatively by means of absorption photographs (A2). They used a 0.1 per cent alcohol solution of a "95 per cent ergosterol", m.p. 161° C., containing traces of other sterols ("probably zymosterol"). This was irradiated under commercial nitrogen in a rotating 30 cc. flask, 20 cm. from a 450-watt mercury arc.

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[1] Most workers note peaks in ergosterol near 2600, 2700, 2800 and 2950 Å., but not at 2500 Å.

[2] The full discussion of his paper will come later.

MORRISON AND CLARK'S EXPERIMENTS

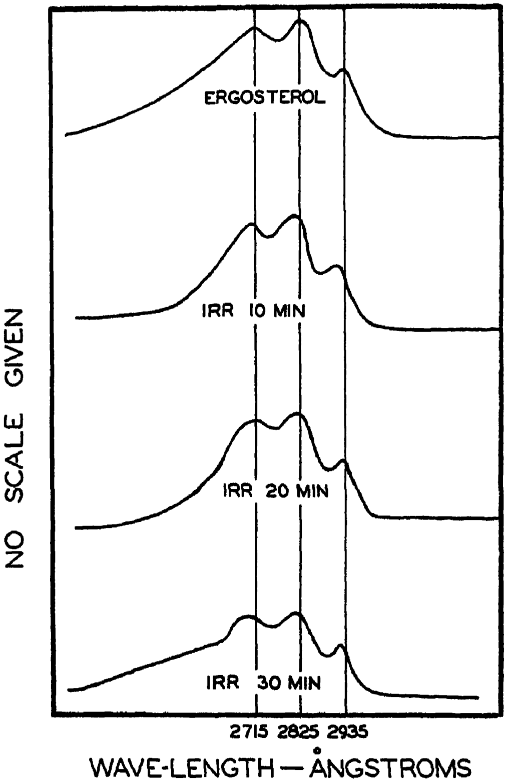
During irradiation, increased absorption was first noticed between 2450 and 2550 Å., one presumes because of development of the band near 2450 Å. When the maximum increase of absorption had been reached, the solutions had a "spectral limit" of 2970 A. Further irradiation resulted in decreased absorption throughout the spectrum, until finally the transparence approximated that of alcohol. They said the changes in an oxygen atmosphere were different, in that the increased absorption in the 2450-2550 Å. region was not noted, but there was instead first a broadening and later a contraction of the 2550-2866 Å. ergosterol absorbing region. The reproduced spectral photographs are so lacking in detail that one can conclude little from their results other than that they probably observed absorption changes in their oxygen-free experiments due to the formation of the band near 2450 Å., and that the changes they observed in the presence of oxygen may have been due to the formation of the band near 2800 Å. which we shall presently discuss.

In France, a number of papers were published about this time dealing in a qualitative or semi-quantitative way with the photodynamics of ergosterol absorption. None of them, however, contributed greatly to the solution of the problem of the absorption of vitamin D.

RESULTS OF THE FRENCH WORKERS

Fabre and Simonnet (99; 1928), for example, found the "optical density" (Ab2) of cholesterol solutions to decrease during early periods of irradiation and later to increase. Similar results were obtained with ergosterol. Because of the known inactivating effects of over-irradiation, they assumed the biological activity to parallel the initial decrease in opacity rather than the later increase in opacity. The latter effect, in fact, was found to be accompanied by the formation of a biologically-inactive, uncrystallizable residue. It is not clear from their paper what wave-length regions were involved in these "opacity changes".

Actual absorption curves of another series of samples (Ab3) (Figure 51), in which the solutions were continuously circulated through a special cell during the photographing of the spectra to prevent over-irradiation by the spectrograph source, showed a progressive diminution of the ergosterol bands and a slow increase of absorption in the short-wave region, presumably due to the formation of the band near 2450 Å. The authors said that parallel biologic and spectrographic tests should be carried out to establish the absorption of the vitamin.



1:10,000 solutions in alcohol

Fig. 51—Absorption of Ergosterol Irradiated With Full Ultra-Violet for Various Times [Fabre and Simonnet (99; 1928)].

ACTIVITY NOT PARALLEL WITH "OPTICAL DENSITY"

Later (100; 1928) Fabre and Simonnet presented these same data, together with the results of biologic tests. The biological activity ceased to increase while the "optical density" (one presumes in the 2700-3100 A. region) was still diminishing, in substantial agreement with the results published independently, about the same time, by Bills, Honeywell, and Cox (95).

In two other papers (101, 102; 1929) these authors reported attempts to correlate the antirachitic activity and the absorption of irradiated ergosterol. For a wide range of exposures, they found all samples to be of approximately the same activity, namely "0.001 mg. per 100 g. of white rat". One

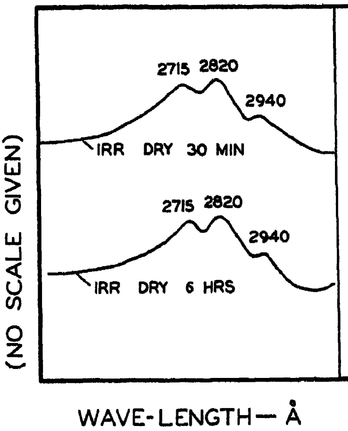


Fig. 52

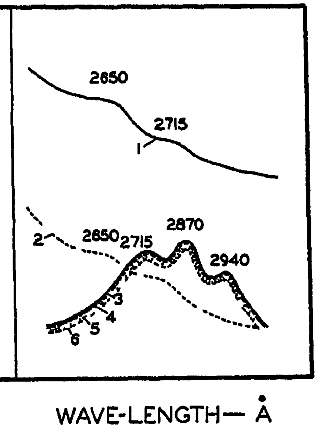


Fig. 53

Fig. 52—Absorption of Ergosterol Irradiated Dry with Full Ultra-Violet [Fabre and Simonnet (101; 1929)].

Fig. 53 -Absorption of Ergosterol Irradiated in Alcohol with Full Ultra-Violet, and of Products of Fractionation of Irradiated Materials with Dilute Alcohol.

Curve 1, solution irradiated 6 hours with air present; curve 2, uncrystallizable residue after fractionation; curve 3, ergosterol before irradiation; curve 4, first crystallized fraction of irradiated material; curve 5, second crystallized fraction; curve 6, third crystallized fraction.

presumes, therefore, that their biological technique was not as accurate as that employed by Bills, Honeywell, and Cox (95), in which the progressive potency changes throughout the period of irradiation could be noted.

BAND NEAR 2450 Å. IN PRODUCTS OF PROLONGED IRRADIATION

The samples used ranged from those in which but a small amount of ergosterol had been converted, as measured by digitonin precipitations, to those in which most of the ergosterol had been changed. In the former class were ether solutions irradiated in an inert atmosphere 20 to 30 minutes and dry ergosterol irradiated 30 minutes to 6 hours. Little absorption change was noted in these products (Figure 52). In the latter class were alcohol solutions irradiated 6 hours "without precautions", which showed marked absorption changes, characterized by a diminution of the ergosterol peaks and a rise in short-wave absorption (Figure 53). "The products intermediate between these extremes," they said, "do not present in any case a biological activity greater than that previously observed, while the absorption bands gradually diminish and there appears a maximum near 2550 Ångströms." Thus to some extent their work offered confirmation of the findings of Bills, Honeywell, and Cox (95) that the 2450 Å. band[1] developed after maximum potency was reached, and could not, therefore, be attributed to the vitamin. As far as their conclusions were concerned, they said simply that "it seems premature to attribute a strictly definite absorption spectrum to the active biological product", pointing out the difficulties due to the absorption of contaminating substances.

By fractionating the irradiated materials with dilute alcohol, they obtained crystalline products similar in absorption and rotation to ergosterol, and uncrystallizable residues, with potencies of the order of that given above, having absorption similar to that of their alcohol solutions irradiated for long times (Figure 53).

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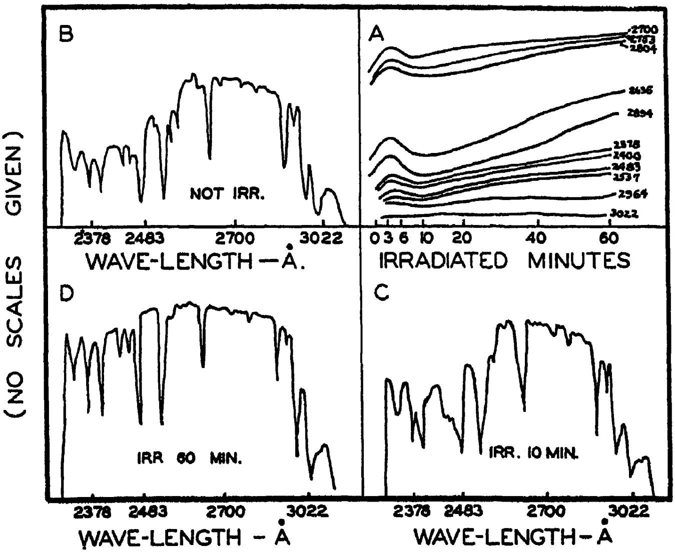
[1] They placed the band at 2550 Å., but one presumes that they observed the same maximum previously noted by other workers.

FURTHER EVIDENCE THAT THE 2450 A. BAND IS NOT DUE TO THE VITAMIN

Two other investigators, Delaplace and Rebière (103; 1929), published results that are difficult to reconcile with any of those that we have discussed. Their curves (A5) of the changes in extinction at various wave-lengths during irradiation[1] indicated (Figure 54, A) that throughout the region 2300-2900 Å. there was first an increase in absorption, then a decrease, and later a prolonged increase. The strange thing is that these changes were closely the same throughout the entire ultraviolet region, and that the trend changed twice at all wave-lengths. One wonders as to the accuracy of their methods. Indeed, a glance at their absorption curves (Figure 54, B, C, D) shows that they bore little relation, except in a very gross way,



1:4000 solutions in alcohol, 5 mm. cell.

Fig. 54—Absorption of Ergosterol Irradiated for Various Times with Full Ultra-Violet, and Curves of Extinction Coefficients Against Time [Delaplace and Rebière (103; 1929)].

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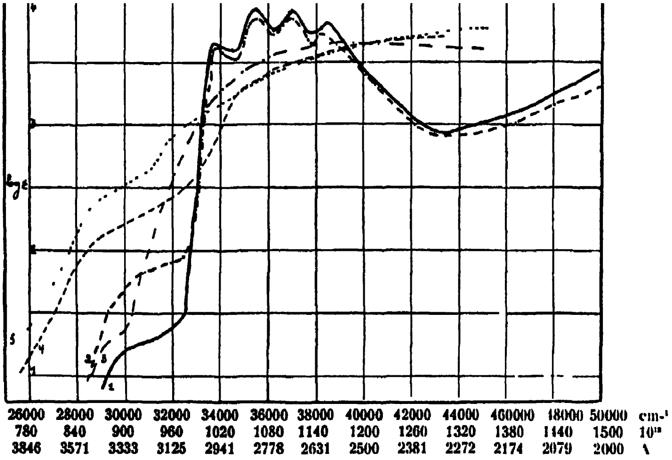
[1] These were obtained by irradiating a 1:4000 solution of ergosterol in alcohol in a 5 mm. cell, 25 cm. from a quartz mercury arc.

to those of other workers. Their absorption curves (B, C, D) showed the usual rise in the short-wave region upon prolonged irradiation, presumably due to the formation of the band near 2450 Å. They cautioned against the acceptance of the short-wave absorption as characteristic of the vitamin, however, because of the evidence against parallelism between this absorption and the activity. Under their conditions, maximum potency was reached after 7 to 10 minutes irradiation, but it was necessary to irradiate 40 minutes to produce pronounced short-wave absorption. These results, then, further confirmed the findings of such previous workers as Smakula (94), and Bills, Honeywell, and Cox (95) regarding the non-identity of the 2450 Å. band with the vitamin.



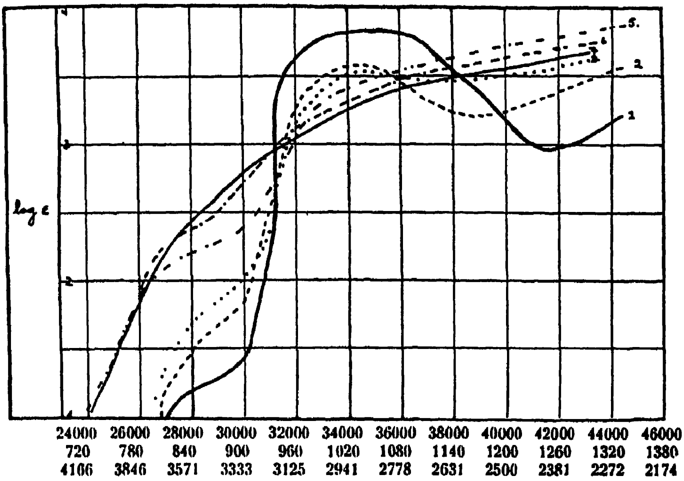
Curve 3, 1/100 normal solution in hexane, curves 4 and 5, 4 g. per L. solutions in hexane-ether (EE).

Fig. 55—Published Absorption Curves of Ergosterol, Ergosterol Acetate, and Irradiated Ergosterol [Castille and Ruppol (104; 1929)].

Curve 1, ergosterol; curve 2, ergosterol acetate; curve 3, ergosterol irradiated in hexane, 1/100 normal, under nitrogen; curve 4, ergosterol irradiated 4 g. per L. in hexane-ether, under nitrogen; curve 5, ergosterol irradiated 60 hours, 4 g. per L. in hexane-ether, air present.

CASTILLE AND RUPPOL'S CURVES

Castille and Ruppol (104; 1929) discussed at some length the absorption changes in irradiated ergosterol, the chemical reactions of the products, and the chemical implications of the absorption changes.[1] Their ergosterol was prepared from ergot and purified through the acetate (m.p. 161.5°; rotation $[\alpha]_D^{20}$ −117° in chloroform). During irradiation, the ergosterol solution was pumped through a closed circuit consisting of a quartz tube mounted in front of a quartz mercury arc, the quartz cells of a spectrograph and polarimeter, and a storage flask into which nitrogen[2] could be introduced and



1 g. per L. solutions in hexane-ether (EE).

Fig. 56 —Published Absorption Curves of Ergosterol Irradiated 4 g. per L. in Hexane-Ether with Air Present [Castille and Ruppol (104; 1929)].

Curve 1, ergosterol; curve 2, irradiated 1 hour; curve 3, irradiated 4 hours; curve 5, irradiated 29 hours; curve 6, irradiated 48 hours; curve 7 irradiated 60 hours. Their tables refer also to curve 4 of this figure, but there is no curve 4 on the figure.

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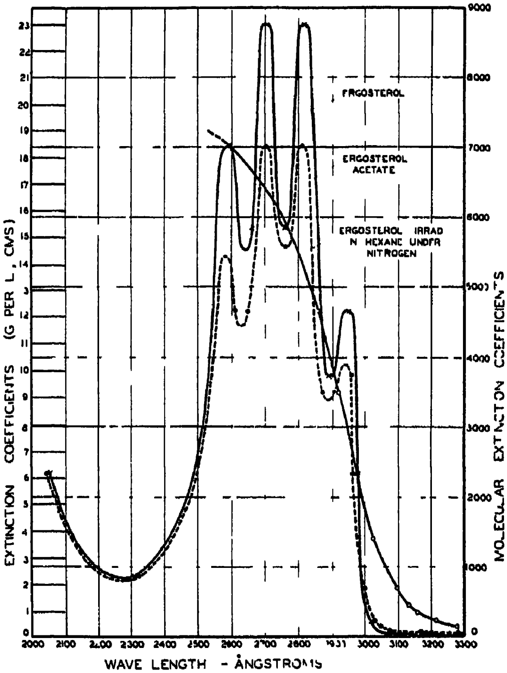
[1] The last two matters will be discussed in a later section.

[2] Freed from oxygen over hot copper and sodium pyrogallate and from water over sulphuric acid.

allowed to leak out slowly. By this arrangement, the optical properties of the solutions could be tested at intervals during irradiation.

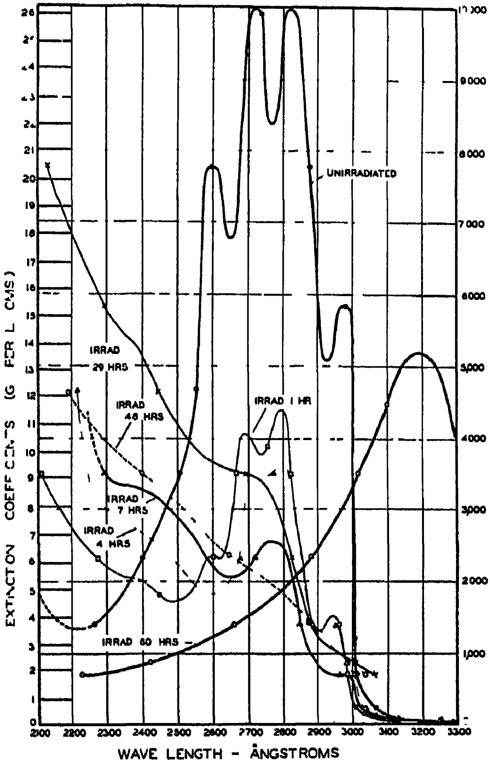
Direct comparison of their absorption curves with those of other workers is difficult because they used a frequency/



Irradiated ergosterol, 1/100 normal solution in hexane (EE).

Fig. 57—Re-plotted Curves of Ergosterol, Ergosterol Acetate, and Ergosterol Irradiated in Hexane under Nitrogen [Castille and Ruppol (104; 1929)].

log-extinction scale instead of the usual wave-length/extinction scale (Figures 55 and 56). Upon plotting curves on the usual basis (Figures 57 and 58) from tables of wave-lengths and extinctions given in their paper, we encountered the strange difficulty that many of the values given in their tables did not correspond at all with those on their curves.[1] Under these circumstances, one does not know what to accept as their true results.



1 g per L. solutions in hexane-ether (EE).

Fig. 58 -Re-plotted Curves of Ergosterol Irradiated 4 g. per L. in Hexane-Ether, Air Present [Castille and Ruppol (104; 1929)].

If one assumes the tables to be correct, the curves of a 4 g. per liter ergosterol solution in 1:1 hexane-ether irradiated with air present (Figure 58) were similar to those obtained by

[1] To cite but one example, their curve of the 60-hour irradiated hexane-ether solution (Fig. 56), gives log ε at 2424 Å. as 3.6, approximately, while the value from their table is 2.94. The corresponding values of ε are 3982 and 871, respectively.

Morton, Heilbron, and Kamm (91), Bills, Honeywell, and Cox (95), and others, except for the slower rate of change.[1] The absorption in the region of the ergosterol peaks diminished first, followed by increased absorption, primarily in the shorter-wave region. One gross deviation stands out the curve of the 60-hour sample as plotted from their tables, which is unlike anything published by anyone else for the absorption of ergosterol irradiation products, and in fact does not agree with their own curve (Figure 56) for this sample. In addition to the discrepancies between the curves and the tables, the lack of agreement between the authors' two curves of ergosterol itself (Figures 55 and 56) raises doubts as to the extent

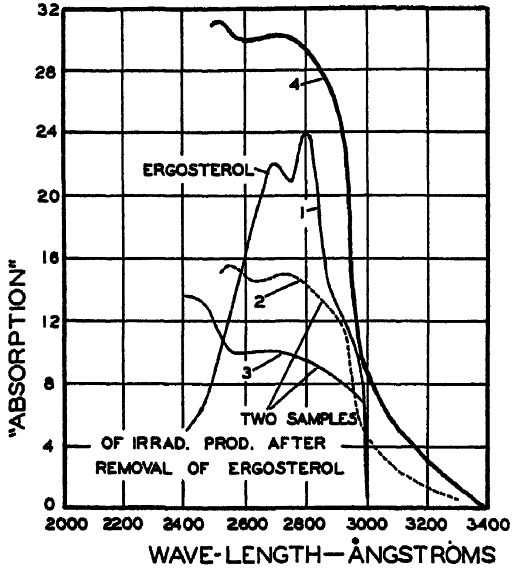


Fig. 59—Irradiation Products After Removal of Unchanged Ergosterol [Webster and Bourdillon (106; 1928)].

For explanation of curve 4, see text.

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[1] One should note, however, that the points were too few and far between to establish the curves properly, so that considerable latitude could be taken in drawing them in.

to which their absorption data may be considered quantitatively.

BAND NEAR 2450 A. PROBABLY DUE TO A DESTRUCTION PRODUCT

Thus closes the chapter regarding the relation of the band near 2450 Å. to vitamin D. The band was observed by many workers—a sufficient number to establish it as characteristic of ergosterol solutions irradiated under certain conditions. Careful studies of the absorption changes, however, indicated it to be due to a secondary reaction product, probably a degradation product of the vitamin, rather than to the vitamin itself. Interest now turned to a new feature in the irradiated ergosterol absorption spectrum, a pronounced band near 2800 Å.

THE BAND NEAR 2800 Å.

A point of view new to the English workers was introduced in a paper presented by Webster and Bourdillon (105; 1928) before the Biochemical Society, at Cambridge, in which they announced that the absorption of ergosterol increased at first upon irradiation, instead of decreasing as found by others.[1] This increase in absorption led them to propose that vitamin D had an absorption band near 2800 Å., a conclusion in agreement with Smakula's suggestion that the vitamin might absorb in the same region as ergosterol.[2] They suggested that three substances were formed in succession: (A) the first, an antirachitically active product with an absorption maximum near 2800 Å., (B) the second, an inactive destruction product with a maximum near 2400 Å., and (C) the third, a final product having neither activity nor appreciable specific absorption.

WEBSTER AND BOURDILLON'S EXPERIMENTS

A detailed description of Webster and Bourdillon's experiments was published later (106; 1928).

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[1] In Germany, Smakula (94) had found a similar increase during the early period (2 to 4 minutes) of irradiation (page 67, Figure 46).

[2] It will be recalled, however, that Smakula attributed two bands to the vitamin, one at 2620 and one at 2930 Å.

Their spectra were not determined accurately;[1] ". . . the curves shown," they said, "are only rough approximations, and no attempt has been made to locate exactly the heads of the individual bands."

In some of the experiments, the unchanged ergosterol was precipitated from the irradiated solutions with digitonin, and the residue was tested spectrographically and biologically. The curves in Figure 59 are of preparations of this type.

INCONSISTENT CURVES FROM ERGOSTEROL-FREE SAMPLES

The spectral photographs of the irradiated ergosterol solutions themselves, before precipitation of the unchanged ergosterol with digitonin, showed increased absorption in the 2540-3130 Å. region during the early periods of irradiation. On the other hand, the absorption curves of the non-precipitated fractions of irradiated solutions treated with digitonin (Figure 59) showed actually less absorption than that of ergosterol except in the region longer than 3000 Å. Nevertheless, the authors said: "This hypothesis[2] is strongly supported by the absorption of the products described above which remain after the removal of ergosterol from an irradiated solution. For, although these products show an absorption which only exceeds that of ergosterol between 300 and 330 $\mu\mu$, if it is assumed that the products are a pure absorbing substance, it is much more probable that the products are a mixture of absorbing and non-absorbing substances. If such non-absorbing substances form 40 per cent of the sample whose absorption is given in curve 2 (Figure 59), the true absorption curve of the absorbing product would be that shown in curve 4 (Figure 59). Such an absorption would satisfactorily account for the increase in absorption shown in the first few minutes of the irradiation of ergosterol." Just what these impurities might have been was not explained. It hardly seems that the hypothesis was "strongly supported" by the curves when one considers that the curves did not agree with it at all, unless transformed in accordance with certain arbitrary assumptions regarding the presence of non-absorbing impurities.

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[1] The spectra were photographed with a quartz mercury arc, and the curves plotted by a method due to Dobson, Griffith, and Harrison. In addition to the curves reproduced here, the paper contains a number of spectrum photographs, which we did not believe it necessary to include.

[2] i. e., that the absorption of the initial product is greater than that of ergosterol.

Irradiation of the products after removal of unchanged ergosterol was found to destroy the absorption. In view of this and the fact that they found all of their biologically potent samples to have high absorption in the region of the ergosterol peaks, they suggested that two substances were formed in succession, the first, vitamin D, with an absorption maximum at 2800 or 2900 Å., the second, a substance with a maximum near 2300 Å.

APPARENT CONFIRMATION OF THE RELATION OF THE 2800 Å. BAND TO THE VITAMIN

In a further note, Webster and Bourdillon (107; 1929) stated that with the assistance of Miss Fischmann and Mr. Jenkins they had "fully confirmed" their hypothesis that ultra-violet irradiation of ergosterol results in the successive production of the three substances "A", "B", and "C", described in their previous papers. They were "convinced" that the first substance ("A") was vitamin D, because on irradiating ergosterol under various conditions and in various solvents and removing the unchanged ergosterol with digitonin, they uniformly found intense absorption between 2700 and 2900 Å. to be accompanied by great antirachitic activity, and smaller absorption by lesser activity. By further irradiating these products through a filter of alcoholic cobalt chloride "(thus excluding wave-lengths less than 2600 Ångströms)" they obtained solutions with slight absorption from 2700 to 2900 Å., but intense absorption at 2400 Å. "These solutions showed no antirachitic activity when tested in doses which would have revealed one five-hundredth of the original activity."

THE POINT OF VIEW AT THE BEGINNING OF 1929

By this time, then, the band near 2450 Å. had come to be regarded as characteristic of a degradation product rather than of the vitamin, and the more favored points of view were those of Smakula in Germany, that bands near 2650 and 2900 Å. might represent the vitamin, and of Webster and Bourdillon in England, that a marked band near 2800 Å. represented it. It seems probable that Webster and Bourdillon's 2800 Å. band was identical with the 2900 Å. band of Smakula. They failed to note his 2650 Å. band, however, possibly for a reason that will soon become evident.

One will recall that Smakula used magnesium spark radiations for irradiating his materials. Since the magnesium spark is very rich in radiations near 2800 Å. and has little energy of shorter wave-lengths, its use was equivalent to employing a filter with a mercury arc in such a way as to eliminate most of the energy of wave-length shorter than 2800 Å. Therein lay the chief difference between the experimental conditions of Smakula and Webster and Bourdillon, and, judging from the experiments about to be described, therein lay the explanation of the finding by Smakula, but not by the English workers, of the band near 2650 Å.

THE BAND NEAR 2650 A.

The importance of the 2650 Å. band was first emphasized by the two Dutch workers Reerink and van Wijk (108; 1929). Prior to their studies, little had been said about the effects of selected spectral regions in irradiating ergosterol, practically all published work having described experiments with the full ultraviolet of the quartz mercury arc. Morton, Heilbron, and Kamm (91) had suggested the use of filtered rays in producing vitamin D. Smakula (94), as we have seen, had made use of a magnesium spark source, which is richest in rays near 2800 Å., and had compared the effects of full and filtered magnesium spark light. Webster and Bourdillon (105, 106, 107) had experimented with the re-irradiation of irradiation products through cobalt chloride filters. None of these reports, however, gave comparative data on the absorption changes produced by the so-called short-wave and long-wave regions of the ultraviolet spectrum. It remained for Reerink and van Wijk to publish such investigations. The widely different results which they obtained by using different spectral regions led many workers to turn for the first time to a serious consideration of the effects of the spectral energy distribution of the activating radiations.

"LONG-WAVE" AND "SHORT-WAVE" RADIATIONS

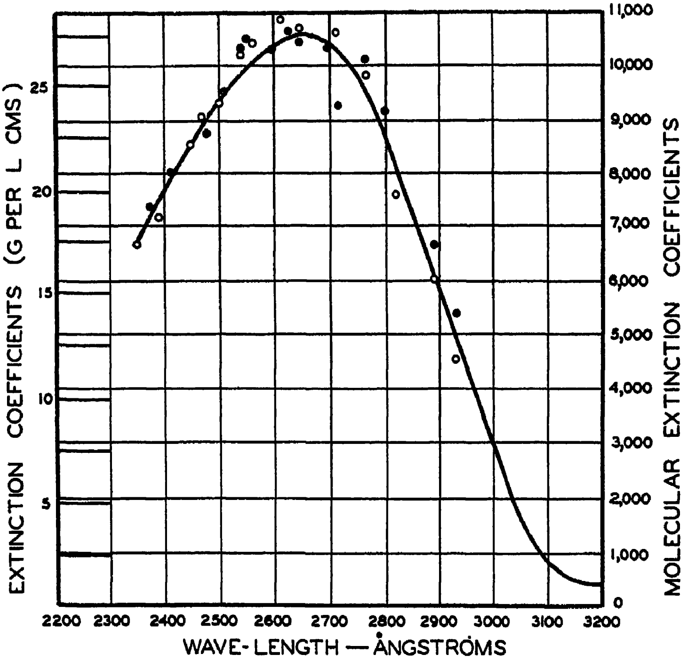
The activating radiations used by them included two broad regions of the ultra-violet spectrum, which they designated as "long-wave" and "short-wave" radiations. The "long-waves" comprised rays from a quartz mercury arc or condensed magnesium spark filtered by 0.5 cm. of 10 per cent benzene solution. This filter, they said, cut off all radiations shorter than 2750 Å. without weakening the longer radiations appreciably. The "short-waves" comprised rays from a mercury column in argon, so operated as to give a strong resonance line at 2537 Å., but weak lines elsewhere in the spectrum, in conjunction with two filters, one a 2 cm. column of saturated chlorine vapour (6 atm. pressure at 15°), which transmitted about 90 per cent at 2540 Å., but absorbed longer wave-lengths "very strongly"; the other, a 0.5 cm. cell of 0.5 per cent solution of KNO_3 in distilled water, which absorbed "all radiations shorter than 241 $\mu\mu$" with other radiations "scarcely weakened". The chlorine filter transmitted, they said, less than 0.1 per cent between 2750 and 3900 Å.

In their early experiments, they irradiated hexane solutions of ergosterol in glass trays with quartz covers, flowing argon over the solutions, but changes in the spectra were apparently caused by traces of oxygen present, so they turned to the use of quartz containers filled with the solutions, cooled in solid carbon dioxide, evacuated with a high vacuum pump, and sealed off. The hexane-ergosterol solutions were agitated during irradiation by an iron stirrer operated by an electromagnet. The spectra were photographed (Ae5) without opening the containers. The ergosterol used was prepared from yeast and purified through the acetate, m.p. 163° in air, 165° in vacuum.

The "long-wave" and "short-wave" irradiations produced very different absorption changes.

EFFECTS OF "LONG-WAVE" RADIATIONS

In their "long-wave" irradiations, they noted increased absorption throughout a large part of the spectrum during the first fifteen minutes of irradiation. This was followed by a diminution of absorption in the long-wave region with but little change in the short-wave region. The 2820 A. maximum diminished from the beginning, while that at 2715 Å. increased at first, proving "conclusively" that the first reaction product had a higher absorption index than ergosterol at 2715 Å. and a lower one at 2820 Å.

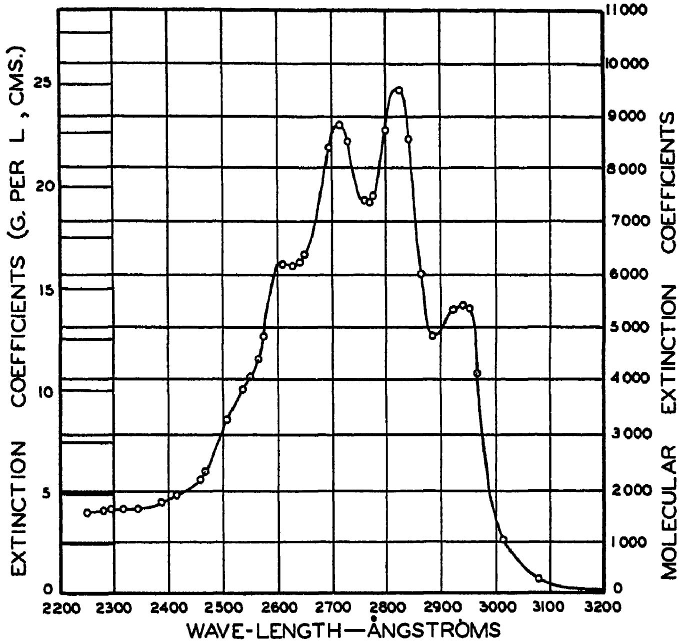


(AAaaI).

Fig. 60—Product "L" of "Long-Wave" Irradiation, as Calculated by the Difference Method and as Measured from the Residue After Removal of Unchanged Ergosterol [Reerink and van Wijk (108; 1929)].

The circles and dots represent values calculated from two different irradiated solutions. The line represents the measured absorption curve of the residue after removal of unchanged ergosterol.

The variations in the curves as irradiation progressed indicated that several substances were being formed successively. To determine the absorption of the first substance or substances,[1] they irradiated solutions for such short periods that most of the ergosterol remained unchanged. By the difference method first applied by Pohl (12) and Smakula (94), that is, by subtracting from the absorption curves of these solutions the absorption of the fractions of unchanged ergosterol remaining in the solutions (determined in their case by digitonin precipitations), they obtained the absorption coefficients of the first reaction product (Figure 60, dots and circles).



0.08 g. per L. solution in hexane (AAaaI).

Fig. 61—Absorption of Ergosterol [Reerink and van Wijk (108; 1929)].

They found that direct measurement of the absorption of the residue after precipitation of the unchanged ergosterol with digitonin yielded reliable results only if every trace of oxygen were excluded. By performing all operations in a metal box, in an atmosphere of nitrogen and hydrogen carefully freed from oxygen, they obtained a spectrum of the first reaction product (Figure 60, unbroken line), practically identical with that given by the difference method.

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[1] They stated that more than one substance might have been formed from ergosterol during the early stages of irradiation.

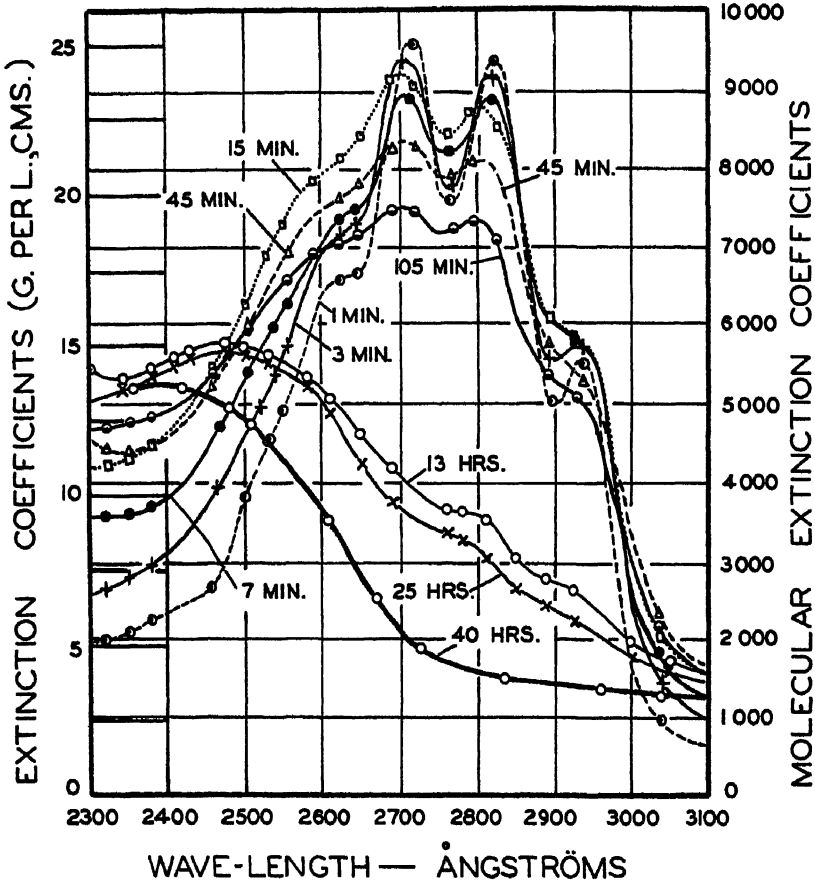


Fig. 62—Absorption of "Long-Wave" Irradiated Ergosterol Solutions [Reerink and van Wijk (108; 1929)].

MAXIMUM AT 2650 Å. CHARACTERIZES THE "LONG-WAVE" PRODUCT, "L"

One notes that their "first reaction product", which they designated "L", was characterized by a single, broad absorption band, with a maximum near 2650 Å. "This product," they said, "will be shown later to be vitamin D. It can be obtained in a crystalline state, but the melting point of the crystals is well below zero degrees. At room temperature the substance has the appearance of a colorless vitreous mass. As the low melting point makes further investigation of the crystals rather difficult, we restrict ourselves at the moment to the statement that the crystals have the appearance of lozenge-shaped plates."

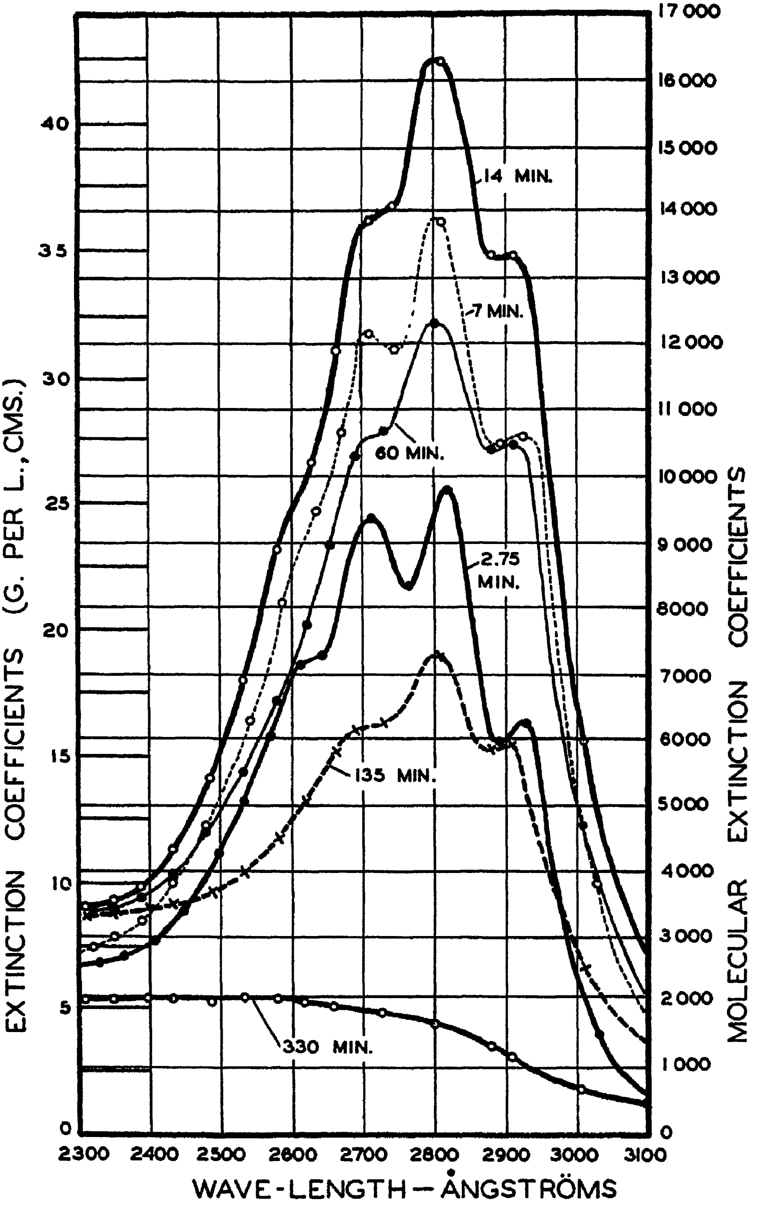
TABLE VIII

Calculated Composition of Ergosterol Solutions Exposed to "Long-Wave" Radiations

Time of Irradiation, Minutes	Ergosterol, Per Cent	Substance "L", Per Cent	Sum Per Cent
1	90 0	10 4	100 4
3	77 1	22 8	99 9
7	64 8	34 7	99 5
15	48 8	50 5	99 3

". . . it can be shown in the following way that as long as the degree of conversion of the solution does not exceed a certain amount the first reaction product is not destroyed.

"Since for an arbitrary wave-length the absorption indices are now known for ergosterol and for the substance *L*, say α_1 and α_2, the absorption index of an irradiated solution, say α_i, must satisfy the condition: $\alpha_i = x\alpha_1 + y\alpha_2$ in which x and y represent the relative amounts of unchanged and changed ergosterol respectively. This is true as long as no secondary reaction takes place. As analogous equations hold for all wave-lengths (with other values of α, but with the same x and y), we can write down a system of equations that must be satisfied simultaneously by the same values for x and y. By applying this procedure to the absorption curves . . . , and solving for x and y by the method of the least squares, we found the figures given in Table VIII."



0.08 g. per L. solutions in hexane (AAaaI).

Fig. 63—Absorption of "Short-Wave" Irradiated Ergosterol Solutions [Reerink and van Wijk (108; 1929)].

"L" PRACTICALLY THE ONLY PRODUCT IN EARLY STAGES OF "LONG-WAVE" IRRADIATION

If substance "L" were vitamin D, the table indicated that up to 15 minutes practically the entire amount of changed ergosterol (50.5 per cent) was the vitamin. Values calculated for times greater than 45 minutes and up to 40 hours showed considerably greater decrease below totals of 100 per cent, indicating the formation of larger quantities of the secondary products, with less absorption in the region neighboring 2700 Å.

After 25 hours' irradiation, the 2800 and 2900 Å. ergosterol bands had practically disappeared, and after 40 hours they had disappeared completely, while a broad band had become prominent between 2400 and 2500 Å. The latter band, speculations about which have been discussed at length, was much more marked when oxygen was not excluded, in agreement with the findings of Bills, Honeywell, and Cox (95). Further photochemical changes took place only very slowly.

EFFECTS OF "SHORT-WAVES"

In the case of the "short-wave" irradiations (Figure 63), Reerink and van Wijk could not calculate consistent spectra for the first irradiation product by the difference method, because secondary reactions apparently took place rapidly from the start.[1] They found the general character of the absorption changes to be: first, an increased absorption throughout the entire range, including 2800 Å., followed by a gradual diminution of absorption to practically complete transparence after 330 minutes. "The most striking features . . . are the gradual disappearance of the band at 262 $\mu\mu$, and the large increase of the bands at 282 $\mu\mu$ and 293 $\mu\mu$ with respect to that at 271.5 $\mu\mu$."

After a considerable period of irradiation, throughout which the nature of the absorption curves continually changed, a stage in the photochemical process was reached in which there appeared a greater degree of regularity and in which succeeding curves seemed to represent different concentrations of the same reaction product. Thus, the ratios of the extinction coefficients of the 14, 60, and 135 minute samples were found to be independent of wave-length within a wide range, indicating these curves to be representative of various concentrations of the same substance. The authors designated this substance, which was apparently characterized by marked absorption at 2800 Å., by "S". Upon prolonged irradiation, it was finally converted into a practically transparent end-product, as illustrated by the curve of the 330 minute sample.

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[1] The intensity of the source was, of course, different, so that the reaction rate would not have been expected to be the same even had the changes obtained been of the same type.

THE PRODUCT "S" OF "SHORT-WAVE" IRRADIATION

The 14 minute curve was chosen as typical of the absorption of "S". This gave the approximate[1] extinction coefficients of "S" at various wave-lengths. Those of "L" (Figure 60), of ergosterol (Figure 61), and of the various "short-wave" irradiated solutions (Figure 63) were known. From these data, the relative concentrations of substances "L" and "S" in the various "short-wave" irradiated solutions were calculated by the method of least squares.[2] As a check, theoretical absorption curves of the 2.75 and 7 minute samples, and for shorter periods of irradiation, were calculated from the concentrations of the various substances deduced to be present in these solutions, and were found to agree closely with the experimental curves. The relative amounts of "L" and "S" calculated by this method to be in the various "short-wave" irradiated solutions indicated that:

> (a) Early in the "short-wave" irradiation, mainly substance "L" was formed, and but little "S".
>
> (b) The rate of destruction of "L" by "short waves" was much greater than that of "S".

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[1] The exact concentration was not known, of course.

[2] The equations for the extinction coefficients were, of course, of the same type as those given above for the "long-wave" irradiated solutions, except that they contained another term to account for the absorption of "S", viz.: $a_j = xa_1 + ya_2 + za_3$, in which "x" represents the amount of unchanged ergosterol, "y" the amount of "L", and "z" the amount of "S".

(c) The concentration of "L" never reached a high percentage in the "short-wave" irradiations.

Special experiments were said to have shown that "S" was destroyed readily by "long-wave" irradiation.

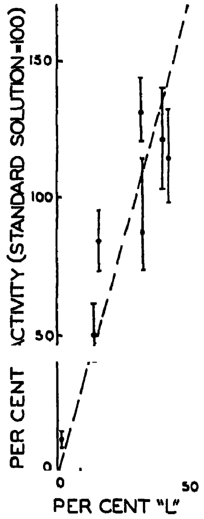

Fig. 64—Relation of Biological Potency of Reerink and van Wijk's "Long-Wave" Irradiated Ergosterol Solutions to Per Cent of "Long-Wave" Product "L", as Estimated Spectrographically [Everse and van Niekerk (109; 1931)].

The dots represent the mean experimental points. The lines through the dots represent the experimental variations.

HIGH ANTIRACHITIC POTENCY OF "L"

Animal experiments carried out by Professors van Leeuwen, Everse, and van Niekerk, they said, showed the antirachitic activity to be closely correlated with the amount of "L" present, as calculated from the spectra. Hence "L" was concluded to be vitamin D. Minimum daily doses of 0.00001 mg. of the most potent sample ("long-wave" irradiated), which was assumed from the degree of transformation to be 50 per cent vitamin D, cured rachitic rats in two weeks. "Short-wave" irradiated samples had activities of 0.1 or less as compared with "long-wave" samples.

The curves of greatly increased absorption found by Webster and Bourdillon, they said, were probably characteristic of the secondary product ("S") because of the action of the short-wave light used in their experiments. They explained the conclusion of Kon, Daniels, and Steenbock (110; 1928)[1] that all wave-lengths are equally effective in forming vitamin D, on the basis that they had exposed samples for too short a time and with too much ergosterol to obtain any change but the formation of the first irradiation product.

EVERSE AND VAN NIEKERK'S BIOLOGIC TESTS OF "L"

The details of the animal experiments with Reerink and van Wijk's samples were eventually published by Everse and van Niekerk (109; 1931). As stated by Reerink and van Wijk in their articles, the findings showed a close proportionality between biological activity and the amount of "L" present in the samples. The accompanying curve (Figure 64) summarizes Everse and van Niekerk's results with these materials.

FURTHER DISCUSSION OF THE 2800 Å. BAND

These experiments afforded strong evidence that the 2900 Å. band of Smakula and the 2800 Å. band of Bourdillon and Webster were not due to vitamin D, but to some other irradiation product. The English workers, however, were still investigating the 2800 Å. band.

Bourdillon, Fischmann, and Jenkins (110; 1929) published further studies of the previously discussed substances "A", "B", and "C" of Webster and Bourdillon.

They irradiated a 0.1 per cent alcohol-ergosterol solution 18 cm. from a quartz mercury arc (125 v., 2.5 amp.). Fifteen cc. of this solution was put in a 22 cc., circular, flat-sided silica cell, 5 cm. in diameter and 1 cm. thick, the remaining space being filled with nitrogen. The cell was rotated during irradiation once per second in the plane of its polished sides, so as to stir the contents. Stirring, it was pointed out, was necessary in order to prevent the formation of a layer of the first irradiation product on the side toward the lamp, this layer preventing the formation of the first product in succeeding layers, and itself being destroyed quite rapidly. In most of their previous work, they said, they had stirred the

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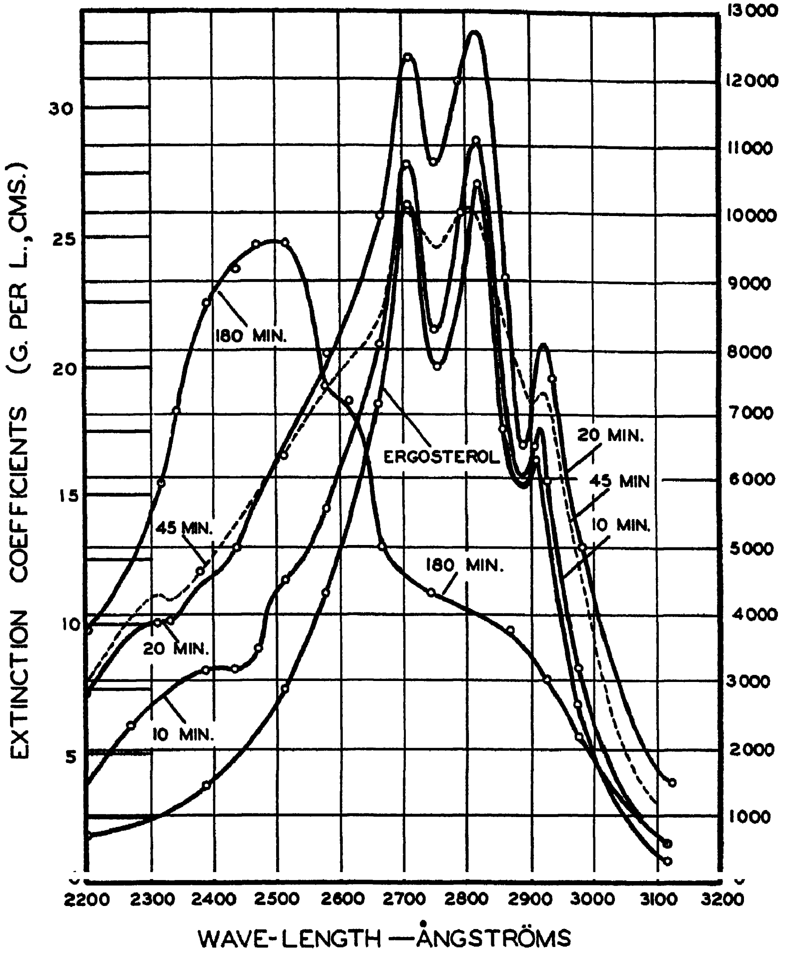
[1] Discussion of this paper will be deferred until later.



0.1 per cent solutions in absolute alcohol (BBaaII).

Fig. 65 —Absorption of Ergosterol Irradiated with Full Ultra-Violet for Various Lengths of Time [Bourdillon, Fischmann, and Jenkins (110; 1929)].

liquid with a stream of nitrogen, but difficulties in that technique led them to use the rotation method in these experiments.

Portions of the solution were removed from the cell at intervals for spectral photographs, and returned to the cell later, the free space being re-filled with nitrogen before continuing the irradiation.

FINDING OF PRONOUNCED ABSORPTION AT 2800 Å

Their absorption curves (Ab5) are shown in Figure 65. For times up to 20 minutes, the absorption increased throughout the entire ultra-violet region, after which it decreased in the 2500-3100 Å. region and simultaneously increased in the 2200-2500 Å. region. If the solution was not stirred, the absorption did not reach so high a value at 2800 Å.

They believed that other workers had not noted this increased absorption at 2800 Å. either because they had not measured the absorption during the earlier stages of irradiation, or because their solutions had not been stirred.[1] Various solvents and various samples of ergosterol were tried with the same results. Differences in concentration had no influence except on the rate of change; at 0.1 per cent., maximum absorption at 2800 Å. was reached in 30 minutes, and at 0.005 per cent., in 2 minutes. "Stirring an alcoholic solution by a stream of oxygen, instead of nitrogen . . . caused no obvious alteration in these early changes in absorption." The results "clearly indicate the formation of a new substance showing intense absorption between 310 and 250 mμ, and itself fairly rapidly destroyed by light."

SHORTER WAVES FAVOR PRODUCTION OF "A"

A 1 cm. filter of 5 per cent alcoholic cobalt chloride solution, which cuts off "all radiation of wave-length shorter than 265, caused a marked decrease in the formation of" the substance of high absorption, suggesting that the short waves "favour the production of *A* rather than its destruction".[2]

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[1] According to a footnote in their paper, an abstract of Smakula's work came to their attention after their paper had gone to press.

[2] This agreed with Reerink and van Wijk's observations concerning their substance "S".

No increase in absorption could be noted when the radiations were filtered through thick plate glass.[1]

In this work, one had, then, apparent confirmation of the hypothesis of the English workers that vitamin D was characterized by a marked absorption band at 2800 Å., more pronounced than that of ergosterol. Their repeated findings caused some to doubt the conclusions of Reerink and van Wijk (109) from their "long-wave" irradiation product that the vitamin had less absorption at 2800 Å. than ergosterol, and was characterized by a broad band with a maximum near 2650 Å. and an extinction of the order of that of ergosterol.

ABANDONMENT OF THE ENGLISH POINT OF VIEW

This unsettled state of affairs was dramatically changed a few months later by the announcement by Bourdillon, Jenkins, and Webster (111; 1930) that the substance "A" with marked bands at 2700 and 2800 Å. was not vitamin D, as they had previously supposed, but some other irradiation product of ergosterol formed simultaneously with the vitamin. They said that they had obtained preparations of high potency but low absorption at 2800 A., and that by irradiating ergosterol through a 2800 Å. xylene filter, precipitating the unchanged ergosterol, and re-irradiating the active material with short waves, they had found a rise in the absorption at 2800 Å. simultaneously with the destruction of activity. Preparations thus obtained resembled closely their "substance A", but had low antirachitic potency. "The non-identity of the two substances," they said, "is in agreement with the findings of Reerink and van Wijk" (108) "and Windaus" (112; 1930).[2]

OTHER INDICATIONS THAT THE VITAMIN BAND IS NEAR 2650 Å

Several other papers published during 1929 and 1930 added testimony to the correctness of Reerink and van Wijk's conclusions.

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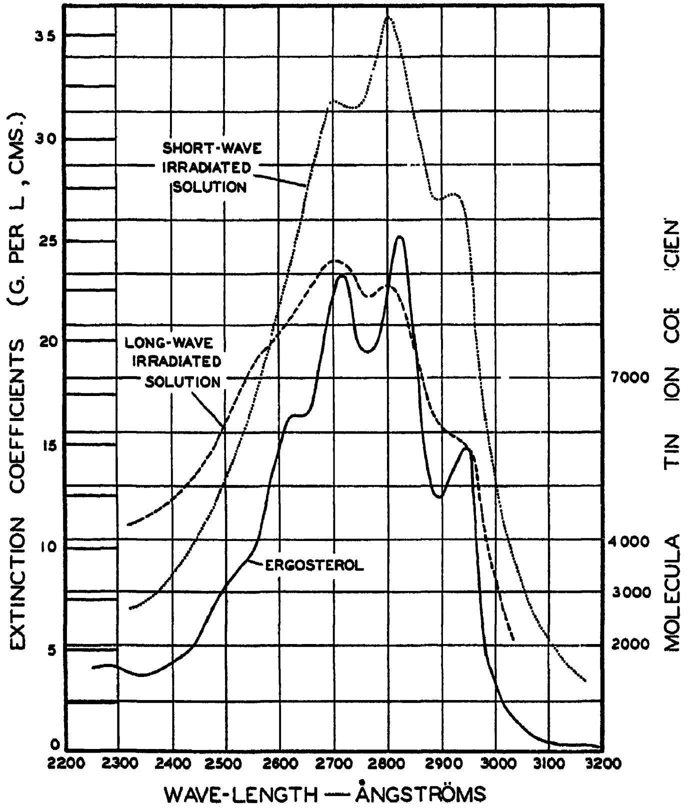
[1] The remainder of their paper is devoted to attempts to fractionate the active principle, which will be discussed later.

[2] This paper will be discussed later.

Holtz (113; 1929) stated in a brief note that the vitamin fraction (after precipitation of the unchanged product with digitonin) of ergosterol irradiated in benzene with the rays of a magnesium spark filtered through Uviol glass had an absorption maximum at 2700 Å. In the sample considered, 96 per cent of the ergosterol had been rendered unprecipitable by digitonin, and the product was active in 0.02γ daily doses. The over-irradiation product formed by using a quartz mer-

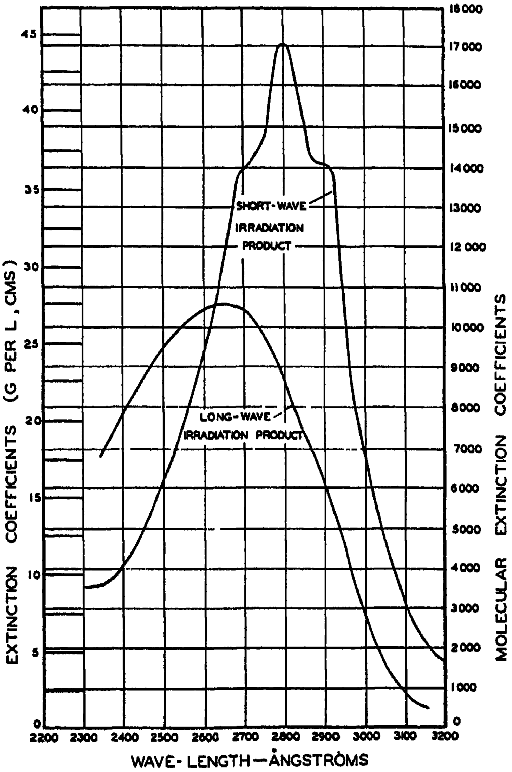


0.08 g. per L. solutions in hexane (AAaaI).

Fig. 66—Absorption of "Long-Wave" and "Short-Wave" Irradiated Ergosterol Solutions [Reerink and van Wijk (116; 1929)].

cury arc to irradiate ether or alcohol solutions of ergosterol in quartz containers had no appreciable absorption between 2300 and 3000 Å.

Windaus in a brief paper on the chemistry of ergosterol and ergosterol derivatives (114; 1929), likewise stated that

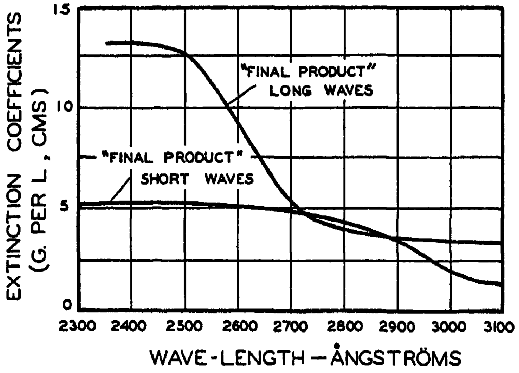


(AAaaI).

Fig. 67 —Calculated Absorption of "Long-Wave" and "Short-Wave" Irradiation Products [Reerink and van Wijk (116; 1929)].

the first irradiation product of ergosterol, "which is spoken of as vitamin D, has its chief absorption at 270 mμ . . . On further irradiation, the characteristic absorption at 270 mμ disappears, and there are formed over-irradiation products which first begin to absorb below 250 mμ . . ."

Reiter (115; 1929) observed that irradiation of ergosterol with full ultra-violet produced marked absorption changes, while the spectrum remained much more constant if the short waves were excluded. These qualitative results were in agreement with those of the Dutch workers.



0.08 g. per L. solutions in hexane (AAual).

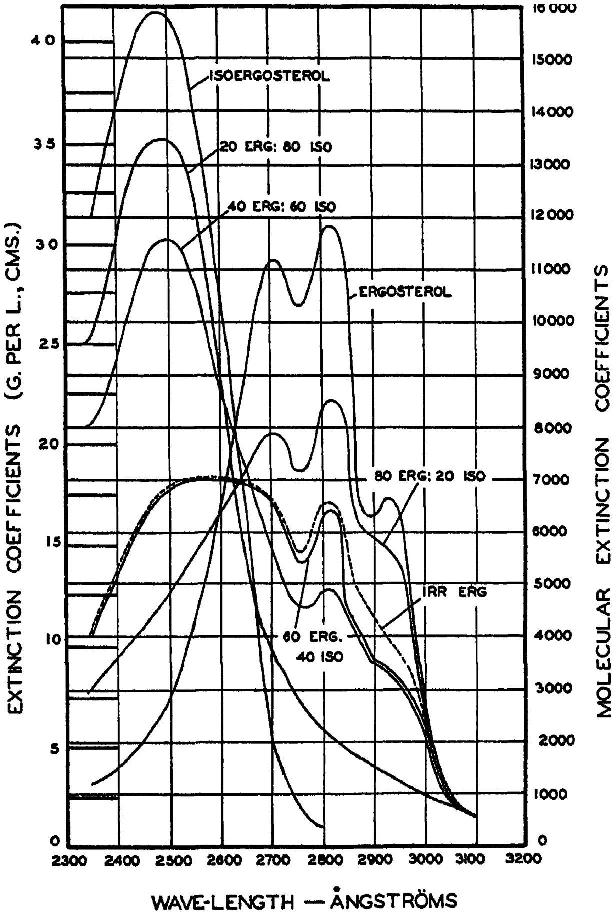
Fig. 68—Absorption of Final Products of "Long-Wave" and "Short-Wave" Irradiation [Reerink and van Wijk (116; 1929)].

FURTHER PAPERS OF REERINK AND VAN WIJK

Reerink and van Wijk themselves published two other papers about this time. The first (116; 1929) discussed the experiments already described, and reproduced curves of the long- and short-wave irradiated solutions (Figure 66), the long- and short-wave reaction products (Figure 67), and the "final products" of long- and short-wave irradiation (Figure 68). Their conclusions are essentially the same as those in their earlier paper (109). The second (117; 1930) is a somewhat more extended account of this work than that which appeared in either of the earlier papers.

OBSERVATIONS OF MARSHALL AND KNUDSON

Some comments of Marshall and Knudson (118; 1930) regarding the spectrum of vitamin D, in their paper on the quantum efficiency of the activation of ergosterol,[1] should probably be noted for the sake of completeness, although they



(CC).

Fig. 69 —Comparison of the Absorption of Irradiated Ergosterol and Various Mixtures of Ergosterol and Isoergosterol [Cox and Bills (119; 1930)].

contribute little of importance to this phase of the vitamin problem. Because they found the potency of ergosterol activated by the 2536 Å. mercury line or full Mg spark light to decrease upon prolonged irradiation with these radiations, they said: "Vitamin D has to absorb light in the same wavelength region as ergosterol in order to be destroyed by light of the same frequency as that which forms it . . . It is evident from these experiments that vitamin D must itself possess a strong absorption band in the same region as ergosterol . . ." The first statement was a necessary conclusion from the principle of conservation of energy, provided the destructive action was a direct effect of the radiations on vitamin D. The observed facts were a necessary but not a sufficient reason for the second conclusion. Ergosterol, for example, was shown by their own experiments to be activated at 2300 Å., yet their absorption curve of ergosterol did not have a "strong absorption band" at 2300 Å. Photochemical effects frequently take place not only in the regions of the absorption maxima but even in regions of minima. Such conclusions could not be accepted as important evidence.

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[1] This paper will be discussed in detail later.

On the whole, therefore, the later work on the absorption of crude irradiation products seemed to support the position of Reerink and van Wijk that the vitamin is characterized by a band near 2650 Å.

FURTHER EVIDENCE CORRELATING THE 2450 Å BAND WITH ISOERGOSTEROL

One will recall that Bills, Honeywell, and Cox (95) and Reerink and van Wijk (96) suggested, from absorption studies, the similarity of the 2450 Å. irradiation product of ergosterol and the isoergosterol of Reindel, Walter, and Rauch (34). Cox and Bills (119; 1930) published absorption curves (Ae6) of mixtures of a "typical" isoergosterol (HCl catalyst, $[\alpha]^{25}_{5461} = -52°$) and ergosterol ($([\alpha]^{25}_{5461} = -157°)$) in various proportions, in comparison with which they showed a curve of "slightly over-irradiated ergosterol"[1] (Figure 69). The similarity of the irradiated ergosterol curve to that of the 60:40 ergosterol-isoergosterol mixture indicated the 2450 Å. band in over-irradiated ergosterol to be due to an isoergosterol. Against this hypothesis was the fact that isoergosterol gave a precipitate with digitonin, while the irradiation product did not. This might have been due, they said, to the latter forming a soluble digitonide, or to its having a difference in the alcohol group . . . "which is known to exert but little influence on sterol absorption bands, but which is concerned in the formation of complexes with digitonin."

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[1] This sample had been exposed 45 minutes to full ultra-violet under the conditions described by Bills, Honeywell, and Cox (95), in which maximum potency of 250,000 average cod-liver oil was reached in 22½ minutes, and by 45 minutes the potency had declined to 140,000.

CONCLUSIONS

We come thus to the end of this section on the absorption changes occurring in ergosterol on irradiation. The papers that we have discussed have included all of those, so far as we know, concerned principally with the absorption changes taking place in ergosterol upon irradiation with ultra-violet, or with the absorption of the crude irradiation products. In considering these papers, we have found vitamin D first credited with an absorption band near 2450 Å., then with a pronounced band near 2800 Å., and finally with a broad band having its maximum near 2650 Å. The band near 2450 Å. was observed by many workers, but the preponderance of evidence showed it to be due to an inactive degradation product of the vitamin. Its identity with an isoergosterol was suggested.

The evidence presented indicated at least four products of irradiation:

(1) Vitamin D, characterized by a band with a maximum near 2650 Å. (substance "L" of Reerink and van Wijk).

(2) An inactive product formed early in the irradiation process (especially when "short-wave" radiations were used) characterized by a pronounced band with a

maximum near 2800 Å. (substance "A" of Webster and Bourdillon, and the short-wave product "S" of Reerink and van Wijk).

(3) An inactive product, presumably a destruction product of the vitamin, characterized by a band, with a maximum near 2450 Å.

(4) The final degradation product or products, having negligible selective absorption, if any.

For the further development of this matter we must turn to a consideration of papers primarily concerned with the fractionation of the irradiation products and the isolation of pure vitamin D. This we shall do in one of the succeeding sections.

DISCUSSION

The following are excerpts from communications regarding the sections of the review already published. Corrections and criticisms for publication in this section are invited.

Doctors Koch, Koch, and Lemon have kindly sent the following discussion of the review of their work (31, 32—pages 147-152, inclusive, vol. 3, this journal):

We shall attempt to answer the chief criticisms of our work by Professor Loofbourow by referring to each statement in turn.

1. p. 143 footnote [this footnote refers to the paper of Bills, Honeywell, and MacNair (22) Ed.]: ". . . the authors obtained the same spectrographic results in examining ergosterol whether they put it in solution alone or dissolved it (in identical concentration) in a 3 per cent. solution of thrice bromine-treated cholesterol, despite the fact that such purified cholesterol is indicated by the experiments of others to have appreciable general absorption."

No sample of cholesterol purified by us either by bromine or potassium permanganate treatment and subsequent repeated recrystallizations from either acetone or alcohol showed *any* general absorption in the region of the ergosterol band. The dibromide of cholesterol does show general absorption.

2. p. 147: (Koch, Koch, and Ragins found) "that purified cholesterol is still activatable, and further that it may be made still more activatable by heating under the proper conditions." Footnote: "Though never more so than unpurified cholesterol."

Does Professor Loofbourow wish to infer in this footnote that the vitamin D activatability or, in other words, the ergosterol-content of the commercial cholesterol was unchanged by the various steps in purification by bromine and potassium permanganate; and further that by such purification activation of the ergosterol is prevented until the cholesterol has been heated? This is obviously not true since the spectrographs show the ergosterol bands clearly in the commercial product and no absorption, either general or specific, below 2350 Å. in the purified product.

3. p. 150: "Further, if instead of comparing Figure 14 *d* with the ergosterol spectra, one compares instead Figure 16 *h* (which is apparently a spectrogram of the same material in the same concentration), one sees that the closest match is obtained with the spectrogram of the 0.008 per cent ergosterol sample."

We have carefully refrained from comparing exposures on one plate with exposures on another plate. The spectrograph was in use for several types of experiments and the difficulty of duplicating exactly all adjustments of slit-width, distances, line-up, etc., made comparisons between spectrographs which were not exposed on the same plate of little value. Whenever a new preparation was compared with previous samples, exposures of the two materials were made under identical conditions. In drawing our conclusions, we have never assumed such a degree of accuracy for the absorption spectra as Professor Loofbourow suggests.

4. p. 150: "as further evidence of the inaccuracy of the spectrographic methods involved - - 'The very striking change in the absorption of purified cholesterol when heated and also the great similarity in the heated cholesterol spectrum and that of Fraction II [the emulsion layer fraction] both highly activatable preparations, to that of ergosterol, at least offered some confirmation to our biological reactions, described in the previous paper'."

This quotation as made is misleading. It was taken from the discussion of the first plate developed, not from the final observations and conclusions as one might be led to suppose. We have described the relatively high activatability of certain cholesterol preparations and are seeking to find in the absorption spectra of these materials some explanation of this fact. The observation that absorption occurred in the same region as for ergosterol encouraged us to continue the spectrographic studies. The final discussion and conclusions given in the paper clearly state that where ergosterol shows specific absorption, these cholesterol preparations show general absorption and for that reason we ascribe the activatability of these latter preparations to something other than ergosterol.

It is probably unwise to include in a report of work, the various states by which the experimenter arrives at his conclusions since by doing so he risks just such misinterpretations.

5. The real crux of Professor Loofbourow's criticisms of the work of Bills, Honeywell, and McNair, and of Koch, Koch, and Lemon is:

a. That the spectrographic methods used are not sufficiently sensitive (rather than not sufficiently accurate) to detect such minute traces of ergosterol as may be biologically important.

b. "It is possible that the general absorption of materials other than ergosterol in their heated samples—an absorption greatest in the shortwave region—acting as a filter, resulted in the irradiation of the ergosterol present under better conditions for activation, so that the irradiated ergosterol in these samples was actually of higher potency than that in the samples of unheated irradiated cholesterol."

We admit this may be true, although Professor Loofbourow presents no evidence in support of his theory. In such case, however, the ergosterol present as a contaminant in our purified cholesterol would necessarily have been activated to a degree as great as, or greater than, that reported for vitamin D preparations recently described (i. e., 0.04 to 0.07γ). Ergosterol mixed with our purified cholesterol could easily be detected spectrographically in the proportion of 1 to 1000. In fact, it could still be detected after this mixture was diluted five times. We were repeatedly able to obtain a ++ cure with dosages of 0.05 to 0.1 mgm. of our heated, purified cholesterol. If ergosterol were present in these preparations in the ratio of 1:1000, then the ergosterol fed with such dosages of cholesterol would have been 0.05 to 0.1γ. But such amounts could readily have been detected spectrographically in the unheated material.

Other evidence reported in the paper of Koch, Koch, and Ragins does not support Loofbourow's thesis. For example, purified cholesterol heated 30 minutes in the presence of $CuSO_4$ and of soda lime both showed marked *general* absorption but the activatability was only slightly increased in the soda lime sample and not at all in the $CuSO_4$ sample. In this case one must conclude either that the general absorption was not an aid to the activation of the ergosterol or that the ergosterol, to which Professor Loofbourow wishes to ascribe all pro-vitamin activity, was partially destroyed by the $CuSO_4$ and soda-lime treatment. But that is simply arguing in a circle. Other more recent studies confirm our earlier statement that whereas increased activatability is always associated with general absorption, the reverse is not true. Marked general absorption is often present without increased activatability.

It still seems to us that the experimental evidence supports our suggestion that cholesterol and possibly all sterols are capable of assuming a configuration which is activatable. This is certainly in harmony with the present conception of the chemist; i. e., that chemical properties are associated with structure and that related compounds have similar properties differing only in degree.

February 18, 1932

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H. B. LEMON.

Dr. Stanislaw Kon has kindly called attention to two errors in the first section of the review (pages 101-156, vol. 3, this journal). Quoting from his letter:

"As far as my own work has been reviewed I have no comments to offer, with the exception that in the citation on page 146, line 11 from top, should read "ultra-violet *light* but not activatable cannot be questioned."

"I would also take the liberty of pointing out one rather unimportant error: On page 120, you state that the paper of Rosenheim and Webster was read before the Biochemical Department of the Imperial College of Science and Technology, London. This is not correct. The paper was read at a meeting of the Biochemical Society, held in the above place."

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